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בעל אמצאה מכח העברה ששמה הוא
Owner, by virtue of Assignment of an invention the title of which is

(בעברית) (Hebrew): חלבון IREN הכנתו והשימוש בו

(באנגלית) (English): IREN Protein, its Preparation and Use

מבקש בזאת כי ינתן לי עליה פטנט
hereby apply for a patent to be granted to me in respect thereof.

• בקשת חלוקה — Application of Division	• בקשת פטנט מוסף — Application for Patent Addition	• דרישה דין קדימה Priority Claim		
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היום 31 בחודש אוגוסט שנת 1999
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לשימוש הלשכה
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IREN PROTEIN, ITS PREPARATION AND USE

חלבון IREN, הכנתו והשימוש בו

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Y/99-71

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Field of the Invention

The present invention relates to DNA sequences encoding a TNF receptor associated (TRAF) protein. More specifically, it relates to cDNA sequences encoding a biologically active protein herein designated IREN and its isoforms capable of binding to TRAF2. The invention also relates to the proteins encoded by the above DNAs, and the use of said proteins and DNA sequences in the treatment or prevention of pathological conditions associated with NF-κB induction, or with any other activity mediated by TRAF2, or with other molecules to which said protein binds.

Background of the Invention

The Tumor Necrosis Factor/Nerve Growth Factor (TNF/NGF) receptor superfamily represents a growing family with over 20 members identified so far in mammalian cells. Although the receptors of this superfamily differ in the primary sequence of their extracellular domains, the TNF/NGF receptor superfamily members share cysteine rich subdomains that are thought to adopt generally similar tertiary folds. (Bazan, 1993; Beutler and van Huffel, 1994; Smith et al., 1994). Except for two receptors, the p55 TNF receptor and Fas/APO1, the various members of this receptor family may have varying structural differences. Nevertheless, there is much similarity of function between the receptors, indicating that they share common signaling pathways. One example for this similarity is the ability of several receptors of the TNF/NGF family to activate the transcription factor NF-κB (see hereinbelow).

TRAF2 is a member of a recently described family of proteins designated TRAF (TNF Receptor Associated Factor) that includes several proteins identified as, for example, TRAF1, TRAF2 (Rothe, M., et al (1994); PCT published application WO 95/33051), TRAF3 (Cheng, G. et al. (1995), TRAF4 (CART1, C-rich motif associated with RING and TRAF domains, Régnier et al. 1995), TRAF5 (Ishida et al. 1996a, Nakano et al. 1996) and TRAF6 (see Cao et al. 1996a, Ishida et al. 1996b). All proteins belonging to the TRAF family share a high degree of amino acid identity in their C-terminal domains, while their N-terminal domains may be unrelated. As shown in a schematic illustration of TRAF2 (Fig. 1 herein), the molecule contains a ring finger motif and two TFIIIA-like zinc finger motifs at its N-terminal end. The C-terminal half of the molecule includes a region known as the "TRAF domain" containing a potential leucine zipper region extending between amino acids 264-358 (called N-TRAF). An additional domain towards the carboxy end of the molecule between amino acids 359-501

(called C-TRAF) is responsible for TRAF binding to the receptors and to other TRAF molecules to form homo- or heterodimers.

Recruitment of TRAF adapter proteins to the cytoplasmic domains of receptor molecules can lead to the assembly of larger signaling complexes that consist of distinct TRAF adapter molecules and other effector proteins with enzymatic functions. Numerous reports have examined the activation of intracellular kinases in response to TRAF-dependent signal transduction. In particular, kinases of the mitogen-activated protein kinase (MAPK) family have been shown to be key players for signaling pathways that are triggered by TRAF-containing complexes. These pathways appear to culminate in c-Jun amino-(N)-terminal kinase (JNK) activation (Reinhard et al. 1997; Song et al. 1997). TRAF proteins can thus serve to modulate the ability of receptors to trigger distinct signaling pathways that lead to phosphorylation and activation of protein kinases and, subsequently, to the activation of transcription factors of the Rel and AP-1 family.

The c-Jun transcription factor is phosphorylated at its amino terminus by JNK, the most downstream member of one MAPK signaling pathway (Hibi et al. 1993). To be activated JNK needs to be phosphorylated by a MAPK kinase (MAPKK, SEK, MEK). This kinase itself is phosphorylated by a MAPKKK (MEKK1), which can be activated through phosphorylation by GCKR (germinal center kinase related) protein, the most upstream kinase described in this pathway (Minden et al. 1994; Lin et al. 1995; Shi and Kehrl 1997). Dominant-negative mutants of either of these proteins that lack kinase activity block TRAF-mediated JNK activation that is induced by members of the TNF/NGFR superfamily. Thus, TRAF proteins appear to regulate the JNK activation pathway at a very proximal step (Liu et al. 1996; Lee et al. 1997; Reinhard et al. 1997). Cells from TRAF2-deficient mice failed to activate JNK in response to TNFα (Yeh et al. 1997). JNK has been demonstrated to mediate the integration of a co-stimulatory signal by CD28 during activation of T lymphocytes (Su et al. 1994). Taken together, these results suggest that co-stimulation by CD28 and TRAF-mediated co-stimulation, after ligation of TNFR-related molecules, utilize the same distal signaling components.

TRAF proteins also appear to play an important role in modulating an early step in receptor-induced activation of NF-κB (Rothe et al. 1995b; Cao et al. 1996; Nakano et al. 1996). NF-κB comprises members of a family of dimer-forming proteins with homology to the Rel oncogene which, in their dimeric form, act as transcription factors. These factors are ubiquitous and participate in regulation of the expression of multiple genes. Although initially

identified as a factor that is constitutively present in B cells at the of Igκ light chain expression. NF-κB is known primarily for its action as an inducible transcriptional activator. In most known cases NF-κB behaves as a primary factor, namely the induction of its activity is by activation of pre-existing molecules present in the cell in their inactive form, rather than its de-novo synthesis which in turn relies on inducible transcription factors that turn-on the NF-κB gene. The effects of NF-κB are highly pleiotropic. Most of these numerous effects share the common features of being quickly induced in response to an extracellular stimulus. The majority of the NF-κB-activating agents are inducers of immune defense, including components of viruses and bacteria, cytokines that regulate immune response, UV light and others. Accordingly, many of the genes regulated by NF-κB contribute to immune defense (see Blank et al., 1992; Grilli et al., 1993; Baeuerle and Henkel, 1994, for reviews).

One major feature of NF-κB-regulation is that this factor can be found in a cytoplasmic non-DNA binding form which can be induced to translocate to the nucleus, bind DNA and activate transcription. This dual form of the NF-κB proteins is regulated by I-κB - a family of proteins that contain repeats of a domain that was initially identified in the erythrocyte protein ankyrin (Gilmore and Morin, 1993). In the unstimulated form, the NF-κB dimer occurs in association with an I-κB molecule which imposes its cytoplasmic localization preventing its interaction with the NF-κB-binding DNA sequence, and activation of transcription. The dissociation of I-κB from the NF-κB dimer constitutes its critical activation step by many of its inducing agents (DiDonato et al., 1995). There is so far little understanding of the way in which cell specificity is determined in terms of responsiveness to the various NF-κB-inducing agents.

Evidence that TRAF proteins can influence receptor-mediated activation of NF-κB came from the demonstration that dominant-negative forms of TRAF2 can inhibit NF- κB activation in response to oligomerization of several TNFR-related molecules, including TNFRII, CD40, CD30, 4-1BB, and Ox40 (Rothe et al. 1994, 1995b; Duckett et al. 1997; Arch and Thompson 1998). However, gene elimination studies in mice have failed to implicate a required role for a specific TRAF in NF-κB activation by any of these receptors (Lee et al. 1997; Yeh et al. 1997). This suggests that receptor engagement may activate NF-κB by more than one pathway.

One of the most potent inducing agents of NF-κB is the cytokine tumor necrosis factor (TNF). There are two different TNF receptors: the p55 and p75 receptors. Their expression

levels vary independently among different cells (Vandenabeele et al., 1995). The p75 receptor responds preferentially to the cell-bound form of TNF (TNF is expressed both as a type II-transmembrane protein and as a soluble protein) while the p55 receptor responds just as effectively to soluble TNF molecules (Grell et al., 1995). The intracellular domains of the two receptors are structurally unrelated and bind different cytoplasmic proteins. Nevertheless, at least part of the effects of TNF, including the cytocidal effect of TNF and the induction of NF-κB, can be induced by both receptors. This feature is cell specific. The p55 receptor is capable of inducing a cytocidal effect or activation of NF-κB in all cells that exhibit such effects in response to TNF. The p75-R can have such effects only in some cells. Others, although expressing the p75-R at high levels, show induction of the effects only in response to stimulation of the p55-R (Vandenabeele et al., 1995). Apart from the TNF receptors, various other receptors of the TNF/NGF receptor family: CD30 (McDonald et al., 1995), CD40 (Berberich et al., 1994; Lalmanach-Girard et al., 1993), the lymphotoxin beta receptor and, in a few types of cells, Fas/APO1 (Rensing-Ehl et al., 1995) are also capable of inducing activation of NF-κB. The IL-1 type-I receptor, also effectively triggering NF-κB activation, shares most of the effects of the TNF receptors despite the fact that it has no structural similarity to them. Novel receptor subunits of the IL-18 receptor complex have been recently cloned and shown to trigger NF-κB translocation and activation in response to IL-18 (Born et al. 1998). The IL-1Rrp as well as a novel protein of the IL-1 receptor family, designated AcPL (<u>Ac</u>cessory <u>P</u>rotein <u>L</u>ike) are both required for IL-18 signaling.

The activation of NF-κB upon triggering of these various receptors results from induced phosphorylation of its associated I-κB molecules. Several components of a specific signal transduction cascade, activated in response to the proinflammatory cytokines TNF-α or IL-1β, have recently been identified. A novel protein kinase designated NIK for <u>N</u>F-κB <u>I</u>nducing <u>K</u>inase was the first to be identified (see co-pending co-owned Patent Application WO 97/37016, Malinin et al. 1996). NIK was found to bind to TRAF2 and to stimulate NF-κB activation. NIK shares sequence similarity with MAP3K kinases and participates in the NF-κB inducing signaling cascade common to receptors of the TNF/NGF family and to the IL-1 type 1 receptor. TNF-α and IL-1β, initiate a signaling cascade leading to activation of two IκB kinases, IKK-1 [IKK-α] and IKK-2 [IKK-β], which phosphorylate IκB at specific N-terminal serine residues [S32 and S36 for IκBα S19 and S23 for IκBβ] (for review see

Mercurio F and Manning A 1999). These kinases were identified as the components of a high molecular weight protein complex designated the IKK signalsome.

Phosphorylated IκB is selectively ubiquitinated by an E3 ubiquitin ligase, the terminal member of a cascade of ubiquitin conjugating enzymes. In the last step of this signaling cascade, phosphorylated and ubiquitinated IκB, which is still associated with NF-κB in the cytoplasm, is selectively degraded by the 26S proteasome. This process exposes the NLS, therefore freeing NF-κB to interact with the nuclear import machinery and translocate to the nucleus, where it binds its target genes to initiate transcription.

The identification of several additional components of the IKK signalsome has given a clue to the potential mechanisms by which receptor activation might be linked to IKK activation. One of these is an NF-κB essential modulator designated NEMO. This murine protein was found to be essential for the activation of NF-κB in a flat cellular variant of HTLV-1 Tax transformed fibroblasts which is unresponsive to all tested NF-κB stimuli (Yamaoka et al. 1998). NEMO was shown to homodimerize and to directly interact with IKK2. The same protein was independently cloned by Kovalenko et al. (see co-pending co-owned Israel Patent Application Nos. 123758 and 126024) as a RIP-binding protein and designated RAP-2. NEMO was later independently cloned by two other groups as a non-kinase component of the IKK signalsome and designated IKKAP-1 (Mercurio F et al 1999b, Rothwarf DM et al 1998). The same protein was also cloned as an E3 interacting protein, which is an adenoviral protein, encoded by the early transcription region and functions to inhibit the cytolytic effects of TNF and was shown to interact with RIP kinase (Li Y et al 1998). These studies provide evidence that NEMO mediates an essential step of the NF-κB signal transduction pathway. Three receptor-associated proteins appear to take part in initiation of the phosphorylation cascade (see diagrammatic illustration in Fig. 2). TRAF2, which when expressed at high levels can by itself trigger NF-κB activation, binds to activated p75 TNF-R (Rothe et al., 1994), lymphotoxin beta receptor (Mosialos et al., 1995), CD40 (Rothe et al., 1995a) and CD-30 (unpublished data) and mediates the induction of NF-κB by them. TRAF2 does not bind to the p55 TNF receptor nor to Fas/APO1, however, it can bind to the p55 receptor-associated protein called TRADD and TRADD has the ability to bind to a Fas/APO1-associated protein called MORT1 (or FADD - see Boldin et al. 1995b and 1996). Another death domain containing serine/threonine kinase receptor-interacting protein, designated RIP (see Stanger et al., 1995) is also capable of interacting with TRAF2 as well as with FAS/APO1, TRADD, the p55 TNF receptor and MORT-1. Thus, while RIP was initially

associated with cell cytotoxicity induction (cell death); its ability to interact with TRAF2 also implicates it in NF-κB activation.

TRAF molecules appear to be involved in the pathway leading to NF-κB activation. These associations apparently allow the p55 TNF receptor and Fas/APO1 to trigger NF-κB activation (Hsu et al., 1995; Boldin et al., 1995; Chinnaiyan et al., 1995; Varfolomeev et al., 1996; Hsu et al., 1996). The triggering of NF-κB activation by the IL-1 receptor occurs independently of TRAF2 and may involve a TRAF2 homologue – TRAF6 and a recently-cloned IL-1 receptor-associated protein-kinase called IRAK (Croston et al., 1995). TRAF6 and IRAK have been also shown to play an important role in IL-18-induced signaling and function (Kanarakaraj et al.1999).

The signaling cascades that are initiated by receptor recruitment of either TRAF molecules or death domain containing adapter proteins are regulated by proteins that can interfere with specific steps by modifying the composition of the multiprotein complexes and/or by blocking protein-protein interactions and downstream effector functions. Several cytoplasmic molecules that bind to TRAFs have been identified. Among them A20, c-IAPs (cellular Inhibitors of Apoptosis), TRIP (TRAF interacting protein) and I-TRAF/TANK (TRAF interacting protein, TRAF family members-associated NF-κB activator). (Rothe et al., 1994; Rothe et al., 1995b; Cheng and Baltimore 1996; Lee et al. 1997; Roy et al. 1997) and two others, one of which is designated clone 9, which shows some sequence homology to the proteins of the present invention, and another designated clone 15 (see co-pending co-owned Patent Application WO 97/37016). Each of these proteins has been shown to be capable at least of binding, and some also of interacting with members of the TRAF family. Yet, the functional roles of these interactions have been demonstrated to be quite distinct. These proteins may be an important link in the ability of TRAF-dependent signal transduction to modulate cell survival. In fact it is not yet clear how TRAFs, trigger the phosphorylation of I-κB. There is also no information yet as to the mechanisms that dictate cell-specific pattern of activation of TRAFs by different receptors, such as observed for the induction of NF-κB by the two TNF receptors. The crystal structure of the TRAF domain of human TRAF has been recently solved (Park, Y.C. et al. 1999). The structure reveals a trimeric self-association of the TRAF domain, which provides an avidity-based explanation for the dependence of TRAF recruitment on the oligomerization of the receptors by their trimeric extracellular ligands.

Accordingly, as regards NF-κB activation and its importance in maintaining cell viability, the various intracellular pathways involved in this activation have heretofore not

been clearly elucidated, for example, how the various TRAF proteins are involved directly or indirectly.

Furthermore, as is now known regarding various members of the TNF/NGF receptor family and their associated intracellular signaling pathways inclusive of various adapter, mediator/modulator proteins (see brief reviews and references in, for example, co-pending co-owned Israel Patent Application Nos. 114615, 114986, 115319, 116588), TNF and the FAS/APO1 ligand, for example, can have both beneficial and deleterious effects on cells. TNF, for example, contributes to the defense of the organism against tumors and infectious agents and contributes to recovery from injury by inducing the killing of tumor cells and virus-infected cells, augmenting antibacterial activities of granulocytes, and thus in these cases the TNF-induced cell killing is desirable. However, excess TNF can be deleterious and as such TNF is known to play a major pathogenic role in a number of diseases such as septic shock, anorexia, rheumatic diseases, inflammation and graft-vs-host reactions. In such cases TNF-induced cell killing is not desirable. The FAS/APO1 ligand, for example, also has desirable and deleterious effects. This FAS/APO1 ligand induces via its receptor the killing of autoreactive T cells during maturation of T cells, i.e. the killing of T cells which recognize self-antigens, during their development and thereby preventing autoimmune diseases. Further, various malignant cells and HIV-infected cells carry the FAS/APO1 receptor on their surface and can thus be destroyed by activation of this receptor by its ligand or by antibodies specific thereto, and thereby activation of cell death (apoptosis) intracellular pathways mediated by this receptor. However, the FAS/APO1 receptor may mediate deleterious effects, for example, uncontrolled killing of tissue which is observed in certain diseases such as acute hepatitis that is accompanied by the destruction of liver cells.

In view of the above, i.e. that receptors of the TNF/NGF family can induce cell death pathways on the one hand and can induce cell survival pathways (via NF-κB induction) on the other hand, there apparently exists a fine balance, intracellularly between these two opposing pathways. For example, when it is desired to achieve maximal destruction of cancer cells or other infected or diseased cells, it would be desired to have TNF and/or the FAS/APO1 ligand inducing only the cell death pathway without inducing NF-κB. Conversely, when it is desired to protect cells such as in, for example, inflammation, graft-vs-host reactions, acute hepatitis, it would be desirable to block the cell killing induction of TNF and/or FAS/APO1 ligand and enhance, instead, their induction of NF-κB. Likewise, in certain pathological circumstances it would be desirable to block the intracellular signaling pathways mediated by the p75 TNF

receptor and the IL-1 receptor, while in others it would be desirable to enhance these intracellular pathways.

Summary of the Invention

It is an object of the present invention to provide a biologically active protein, isoforms, analogs, fragments or derivatives thereof capable of binding to the tumor necrosis factor receptor-associated (TRAF) proteins. As the TRAF binding proteins are involved in the modulation or mediation of the activation of the transcription factor NF-κB, which is initiated by some of the TNF/NGF receptors, as well as others as noted above, the protein according to the present invention by binding to TRAF proteins may therefore be capable of modulating or mediating the intracellular signaling processes initiated by various ligands binding to their receptors. Such ligands are e.g. TNF, FAS ligand and others and modulation/mediation may be e.g. NF-κB activation, via interaction directly or indirectly with TRAF protein (e.g. induction of NF-κB activation by TRAF2 and TRAF6 and inhibition of NF-κB activation, by TRAF3).

The biologically active protein of the invention and its isoforms, analogs, fragments or derivatives may likewise be indirect modulators/mediators of the intracellular biological activity of a variety of other proteins which are capable of interacting with TRAF proteins directly or indirectly (e.g. FAS/APO1 receptor, p55 TNF receptor, p75 TNF receptor, IL-1 receptor and their associated proteins, such as, for example, MORT-1, TRADD, RIP).

Another object of the invention is to provide antagonists (e.g. antibodies, peptides, organic compounds, or even some isoforms) to the above novel TRAF-binding protein, isoforms, analogs, fragments and derivatives thereof, which may be used to inhibit the signaling process, or, more specifically, to inhibit the activation of NF-κB and its associated involvement in cell-survival processes, when desired. Likewise, when the TRAF-binding protein of the invention or the TRAF protein to which they bind (e.g. TRAF3) are themselves inhibitory for NF-κB activation, then it is an object to provide antagonists to the TRAF-binding protein to activate the signaling process or more specifically, to block the inhibition of NF-κB activation and hence bring about enhanced NF-κB activation, when desired.

A further object of the invention is to use the above novel TRAF-binding protein, isoforms, analogs, fragments and derivatives thereof, to isolate and characterize additional proteins or factors, which may be involved in regulation of TRAF protein activity and/or the

above noted receptor activi .g. other proteins which may bind TRAF proteins and influence their activity, and/or to isolate and identify other receptors or other cellular proteins further upstream or downstream in the signaling process(es) to which these novel proteins, analogs, fragments and derivatives bind, and hence, in whose function they are also involved.

A still further object of the invention is to provide inhibitors which can be introduced into cells to bind or interact with the novel TRAF-binding protein and possible isoforms thereof, which inhibitors may act to inhibit TRAF protein-associated activity in, for example, NF-κB activation and hence, when desired, to inhibit NF-κB activation; or which may act to inhibit inhibitory TRAF-associated activity (e.g. TRAF3) in NF-κB activation and hence, when desired, to enhance NF-κB activation.

Moreover, it is an object of the present invention to use the above-mentioned TRAF-binding protein, isoforms and analogs, fragments and derivatives as antigens for the preparation of polyclonal and/or monoclonal antibodies thereto. The antibodies, in turn, may be used, for example, for the purification of the new proteins from different sources, such as cell extracts or transformed cell lines.

Furthermore, these antibodies may be used for diagnostic purposes, e.g. for identifying disorders related to abnormal functioning of cellular effects mediated directly by TRAF proteins or mediated by the p55 TNF receptor, FAS/APO1 receptor, or other related receptors and their associated cellular proteins (e.g. MORT-1, TRADD, RIP), which act directly or indirectly to modulate/mediate intracellular processes via interaction with TRAF proteins.

A further object of the invention is to provide pharmaceutical compositions comprising the above novel IREN protein, isoforms, or analogs, fragments or derivatives, as well as pharmaceutical compositions comprising the above noted antibodies or other antagonists.

The present invention thus provides a novel IREN protein binding to at least TRAF2 and having a high specificity of binding to TRAF2. Hence is a modulator or mediator of TRAF2 intracellular activity. TRAF2 is involved in the modulation or mediation of at least one intracellular signaling pathway being the cell survival- or viability- related pathway in which TRAF2 is directly involved in activation of NF-κB which plays a central role in cell survival.

In fact, this protein, designated IREN (for IκB REgulator) binds to TRAF2 and apparently acts in the NF-κB signalling pathway downstream to NIK but upstream to NEMO

and IKK1 and enhances IKK1 phosphorylation of IκB. Further, TRAF2 by being capable of interaction directly or indirectly with the above noted p55 TNF receptor, p75 TNF receptor, FAS/APO1 receptors and their associated proteins MORT-1, TRADD and RIP, also is a mediator or modulator of the NF-κB induction or activation activity attributed to these receptors. TRAF2 is therefore a modulator/mediator of the cell survival pathways (as opposed to the cell death pathways) mediated by these receptors and their associated proteins and as such the extent of interaction between these receptors and/or proteins with TRAF2 is an important factor in the outcome of the activity of these receptors (once activated by their ligands), namely, whether the cells will survive or die. Accordingly, the proteins of the invention, play a key role in this interaction between TRAF2 and the other proteins/receptors with which TRAF2 interacts, as proteins such as IREN by binding specifically to TRAF2 will modulate its activity and/or will have their activity modulated by interaction with TRAF2.

As will be used herein throughout, TRAF protein activity, for example TRAF2 activity, is meant to include its activity in modulation/mediation in the cell survival pathway, such as NF-κB induction/activation. Likewise, as used herein throughout TRAF-binding protein, in particular TRAF2-binding protein, activity is meant to include modulation/mediation of TRAFs, in particular, TRAF2 activity by virtue of specific binding to TRAFs, especially TRAF2 proteins, this modulation/mediation including modulation/mediation of cell survival pathways, in particular, those relating to NF-κB activation/induction in which TRAF proteins, especially TRAF2 is involved directly or indirectly. Thus IREN may be considered as an indirect modulator/mediators of all the above mentioned proteins and possibly a number of others which are involved in cell survival, such as NF-κB activation/induction and to which TRAF2 (or other TRAF proteins) binds, or with which TRAF2 (or other TRAF proteins) interacts in a direct or indirect fashion. Likewise TRAF2 is involved in the regulation of AP1 transcription factor through activation of the Jun kinase cascade and thus IREN may have a role in the Jun kinase activation pathway or in the control of other gene activation pathways e.g. - the p38 kinase pathway. It thus may have an important role in the control of inflammation and other non-apoptotic effects of TNF as well as in the control of apoptosis.

More specifically, the present invention provides a DNA sequence encoding a protein capable of binding to TRAF selected from:

(a) a cDNA sequence of the herein designated IREN comprising the nucleotide sequence depicted in Fig. 3;

(b) a cDNA sequence of the herein designated isoform IREN-10B comprising the nucleotide sequence depicted in Fig. 4;

(c) a cDNA sequence of the herein designated isoform IREN-E comprising the nucleotide sequence depicted in Fig. 5;

(d) a fragment of a sequence (a)-(c) which encodes a biologically active protein capable of binding to at least the residues 225-501 of the amino acid sequence of TRAF2;

(e) A DNA sequence capable of hybridization to a sequence of (a)-(d) under moderately stringent conditions and which encodes a biologically active protein capable of binding to at least the residues 225-501 of the amino acid sequence of TRAF2; and

(f) A DNA sequence, which is degenerate as a result of the genetic code to the DNA sequences, defined in (a)-(e) and which encodes a biologically active protein capable of binding to at least the residues 225-501 of the amino acid sequence of TRAF2.

Embodiments of the above DNA sequence of the invention encoding the protein encoded by IREN include:

(i) A DNA sequence encoding the protein IREN, its biologically active isoforms, fragments or analogs thereof, capable of binding to TRAF2 and capable of modulating the activity of NF-κB and IREN isoforms, fragments or analogs thereof;

(ii) A DNA sequence as in (i) above, selected from the group consisting of:

a) A cDNA sequence derived from the coding region of a native IREN protein;

b) DNA sequences capable of hybridization to a sequence of (a) under moderately stringent conditions and which encode a biologically active IREN; and

c) DNA sequences, which are degenerate as a result of the genetic code to the sequences, defined in (a) and (b) and which encode a biologically active IREN protein;

(iii) A DNA sequence as in (i) or (ii) above comprising at least part of the sequence depicted in Fig. 3 and encoding at least one active IREN protein, isoform, analog or fragment;

(iv) A DNA sequence as in (iii) above encoding an IREN protein, isoform, analog, or fragment having at least part of the amino acid sequence depicted in Fig. 3.

In another aspect, the invention provides proteins or polypeptides encoded by the above noted DNA, provided that they are capable of binding to TRAF2, preferably to at least the 225-501 amino acid sequence of TRAF2 and the isoforms, analogs, fragments and

derivatives of said protein and polypeptides. Embodiments of these proteins/polypeptides, according to the invention include:

(a) A protein being the protein herein designated IREN:

(b) Isoforms, fragments, analogs and derivatives thereof; and

(c) An IREN protein, isoforms, analogs, fragments and derivatives thereof having at least part of the amino acid sequence depicted in Fig. 6.

In yet another aspect, the invention provides a vector comprising any of the above DNA sequences according to the invention which are capable of being expressed in host cells selected from prokaryotic and eukaryotic cells; as well as transformed prokaryotic and eukaryotic cells containing said vector.

The invention also provides a method for producing a protein, isoform, analog, fragment or derivative encoded by any of the above DNA sequences according to the invention which comprises growing the above mentioned transformed host cells under conditions suitable for the expression of said protein, isoforms, analogs, fragments or derivatives, effecting post-translational modification, as necessary, for obtaining said protein, isoform, analogs, fragments or derivatives and isolating said expressed protein, isoforms, analogs, fragments or derivatives.

In a further aspect, the invention provides antibodies or active fragments or derivatives thereof, specific for the above TRAF-binding proteins, analogs, isoforms, fragments or derivatives thereof or specific for the IREN protein, isoform, analog, fragment or derivative thereof noted above.

In a different aspect, the invention provides the following screening methods:

(i) A method for screening of a ligand capable of binding to a protein according to the invention, as noted above, including isoforms, analogs, fragments or derivatives thereof, comprising contacting an affinity chromatography matrix to which said protein, isoform, analog, fragment or derivative is attached with a cell extract whereby the ligand is bound to said matrix, and eluting, isolating and analyzing said ligand.

(ii) A method for screening of a DNA sequence coding for a ligand capable of binding to a protein, isoform, analog, fragment or derivative according to the invention as noted above, comprising applying the yeast two-hybrid procedure in which a sequence encoding said protein, isoform analog, derivative or fragment is carried by one hybrid vector and sequences from a cDNA or genomic DNA library are carried by the second hybrid vector, transforming

yeast host cells with said v[illegible]s, isolating the positively transfor[illegible]cells, and extracting said second hybrid vector to obtain a sequence encoding said ligand.

Similarly, there is also provided a method for isolating and identifying proteins, isoforms, analogs, fragments according to the invention noted above, capable of binding directly to TRAF2, comprising applying the yeast two-hybrid procedure in which a sequence encoding said TRAF2 is carried by one hybrid vector and sequence from a cDNA or genomic DNA library is carried by the second hybrid vector, the vectors then being used to transform yeast host cells and the positive transformed cells being isolated, followed by extraction of the said second hybrid vector to obtain a sequence encoding a protein which binds to said TRAF2.

In yet another aspect of the invention there is provided a method for the modulation or mediation in cells of the activity of NF-κB or any other intracellular signaling activity modulated or mediated by TRAF2 or by other molecules to which a protein, isoform, analog, fragment or derivative thereof of the invention as noted above, said method comprising treating said cells by introducing into said cells one or more of said protein, isoform, analog, fragment or derivative thereof in a form suitable for intracellular introduction thereof, or introducing into said cells a DNA sequence encoding said one or more protein, isoform, analog, fragment or derivative thereof in the form of a suitable vector carrying said sequence, said vector being capable of effecting the insertion of said sequence into said cells in a way that said sequence is expressed in said cells.

Embodiments of this above method for modulation/mediation in cells of the activity of NF-κB or any other intracellular signaling activity modulated or mediated by TRAF2 or other molecules include:

(i) A method as above, wherein said treating of cells comprises introducing into said cells a DNA sequence encoding said IREN protein, isoform, fragment, analog or derivative in the form of a suitable vector carrying said sequence, said vector being capable of effecting the insertion of said sequence into said cells in a way that said sequence is expressed in said cells.

(ii) A method as above wherein said treating of said cells is by transfection of said cells with a recombinant animal virus vector comprising the steps of:

(a) constructing a recombinant animal virus vector carrying a sequence encoding a viral surface protein (ligand) that is capable of binding to a specific cell surface receptor on the surface of said cells to be treated and a second sequence encoding said IREN protein isoforms, analogs, fragments and derivatives according to the invention, that when expressed

in said cells is capable of modulating/mediating the activity of NF-κB or any other intracellular signaling activity modulated/mediated by TRAF2 or other said molecules; and

(b) infecting said cells with said vector of (a).

Likewise, the present invention also provides a method for modulating TRAF2 modulated/mediated effect on cells comprising treating said cells with the antibodies or active fragments or derivatives thereof, according to the invention as noted above, said treating being by application of a suitable composition containing said antibodies, active fragments or derivatives thereof to said cells, wherein when the IREN protein or portions thereof of said cells are exposed on the extracellular surface, said composition is formulated for extracellular application, and when said IREN protein is intracellular said composition is formulated for intracellular application.

Other methods of the invention for modulating the TRAF2 modulated/mediated effect on cells include:

(i) A method comprising treating said cells with an oligonucleotide sequence encoding an antisense sequence for at least part of the DNA sequence encoding said IREN protein, this DNA sequence being any of the above mentioned ones of the invention, said oligonucleotide sequence being capable of blocking the expression of said IREN protein.

(ii) A method as in (i) above wherein said oligonucleotide sequence is introduced to said cells via a recombinant virus as noted above, wherein said second sequence of said virus encodes said oligonucleotide sequence.

(iii) A method comprising applying the ribozyme procedure in which a vector encoding a ribozyme sequence capable of interacting with a cellular mRNA sequence encoding said IREN protein, isoform, analog, fragment or derivative of the invention noted above, is introduced into said cells in a form that permits expression of said ribozyme sequence in said cells, and wherein when said ribozyme sequence is expressed in said cells it interacts with said cellular mRNA sequence and cleaves said mRNA sequence resulting in the inhibition of expression of said IREN protein in said cells.

In the above methods and embodiments thereof of the invention there is included also a method for the prevention or treatment of a pathological condition associated with NF-κB induction or with any other activity mediated by TRAF2 or by other molecules to which a protein, isoform, analog, fragment or derivative, according to the invention, binds, said method comprising administering to a patient in need an effective amount of a protein, isoform, analog, fragment or derivative, according to the invention, or a DNA molecule

coding therefor, or a molecule capable of disrupting the interaction of said protein, isoform, analog, fragment or derivative, with TRAF2 or any other molecule to which said protein, isoform, analog, fragment or derivative binds. In this method of the invention, said protein of the invention administered to the patient in need can be specifically the protein encoded by IREN, or a DNA molecule coding therefor. The protein encoded by IREN is believed at present to modulate NF-κB induction by IKK-1 and NIK. In an additional aspect of the invention there is provided a pharmaceutical composition for the modulation of the TRAF2 modulated/mediated effect on cells comprising, as active ingredient IREN its biologically active fragments, analogs, derivatives or mixtures thereof.

Other pharmaceutical compositions or embodiments thereof according to the invention include:

(i) A pharmaceutical composition for modulating the TRAF2 modulated/mediated effect on cells comprising, as active ingredient, a recombinant animal virus vector encoding a protein capable of binding a cell surface receptor and IREN, its biologically active isoforms, active fragments or analogs, according to the invention.

(ii) A pharmaceutical composition for modulating the TRAF2 modulated/mediated effect on cells comprising as active ingredient, an oligonucleotide sequence encoding an anti-sense sequence of the IREN mRNA sequence according to the invention.

A further embodiment of the above pharmaceutical composition is specifically a pharmaceutical composition for the prevention or treatment of a pathological condition associated with NF-κB induction or with any other activity mediated by TRAF2 or by other molecules to which a protein, analog, isoform, fragment or derivative, according to the invention binds, said composition comprising an effective amount of a protein, analog, isoform, fragment or derivative, according to the invention or a DNA molecule coding therefor, or a molecule capable of disrupting the interaction of said protein, analog, isoform, fragment or derivative, with TRAF2 or any other molecule to which said protein, analog, isoform, fragment or derivative, binds. In a yet further specific embodiment said pharmaceutical composition comprising an effective amount of the protein encoded by IREN, an isoform, analog, derivative or fragment of IREN, or a DNA molecule coding therefor.

In yet another specific embodiment, the invention provides a pharmaceutical composition for the prevention or treatment of a pathological condition associated with NF-κB induction or with any other activity mediated by TRAF2 or by other molecules to which the protein IREN binds, said composition comprising a molecule capable of interfering

with the activity of IREN. In this composition, the interfering molecule may be an effective amount of IREN mutated in active site residues, this mutated IREN serving to interfere with native IREN.

One known condition associated with NF-κB induction (abnormal) is AIDS, others are e.g. autoimmune diseases, as well as tumors.

Still further aspects and embodiments of the invention are:

(i) A method for identifying and producing a ligand capable of modulating the cellular activity modulated/mediated by a protein, isoform, analog, fragment or derivative, according to the invention, comprising:

a) Screening for a ligand capable of binding to a polypeptide comprising at least a portion of the IREN sequence depicted in Fig. 6.

b) Identifying and characterizing a ligand, other than TRAF2 or portions of a receptor of the TNF/NGF receptor family, found by said screening step to be capable of said binding; and

c) Producing said ligand in substantially isolated and purified form.

(ii) A method for identifying and producing a ligand capable of modulating the cellular activity modulated/mediated by IREN comprising:

a) Screening for a ligand capable of binding to a polypeptide comprising at least a portion of the IREN sequence depicted in Fig. 6.

b) Identifying and characterizing a ligand, other than TRAF2 or portions of a receptor of the TNF/NGF receptor family, found by said screening step to be capable of said binding; and

c) Producing said ligand in substantially isolated and purified form.

(iii) A method for identifying and producing a ligand capable of directly or indirectly modulating the cellular activity modulated/mediated by IREN comprising:

a) Screening for a molecule capable of modulating activities modulated/mediated by IREN;

b) Identifying and characterizing said molecule; and

c) Producing said molecule in substantially isolated and purified form.

(iv) A method for identifying and producing a molecule capable of directly or indirectly modulating the cellular activity modulated/mediated by a protein, isoform, analog, fragment or derivative of the invention, comprising:

a) Screening for a molecule capable of ulating activities modulated/mediated by an IREN protein, isoform, analog, fragment or derivative according to the invention:

b) Identifying and characterizing said molecule: and

c) Producing said molecule in substantially isolated and purified form.

Other aspects and embodiments of the present invention are also provided as arising from the following detailed description of the invention.

It should be noted that, where used throughout, the following terms: "modulation/mediation of the TRAF (or TRAF2) effect on cells" and any other such "modulation/mediation" mentioned in the specification are understood to encompass *in vitro* as well as *in vivo* treatment and, in addition, also to encompass inhibition or enhancement/augmentation.

Description of Figures

Figure 1: shows a diagrammatic illustration of the structure of the TRAF2 molecule.

Figure 2: shows a schematic diagram illustrating some of the proteins involved in NF-κB activation.

Figure 3A shows the nucleotide sequence of IREN's 5-prime UTR (from the beginning of the sequence until ATG with Kozak sequence) which is identical in all 3 IREN splice isoforms.

Figure 3B: shows the nucleotide sequence of IREN

Figure 4: shows the nucleotide sequence of IREN-10B.

Figure 5: shows the nucleotide sequence of IREN-E.

Figure 6: shows the amino acid sequence of IREN.

Figure 7: shows the amino acid sequence of IREN-10B.

Figure 8: shows the amino acid sequence of IREN-E.

Figure 9: shows a comparison between the sequence of IREN and its isoforms IREN-10B and IREN-E.

Figure 10: shows in a diagrammatic fashion results of induction of NF-κB activation by IKK-1, by wild type IREN, NIK and NEMO and mutants thereof.

Figure 11: shows an autoradiogram of FLAG-IKK1, GST-IkappaB and NEMO, obtained after transfection of 293 cells with pcFLAG CHUK (encoding murine IKK1) and pc20.4 (encoding the NEMO protein), together with pcHIS-IRENΔN (pcHIS-IREN $_{198-541}$, left lane), pcHIS-IREN (middle lane), or the empty pcDNA3 vector (right lane) as control. Immunoprecipitation and kinase assays were carried out as described in Example 3. The sizes of the visible bands correspond to the molecular weights determined for FLAG-IKK1, GST-IkappaB and NEMO.

Detailed Description of the Invention

The present invention concerns a cDNA sequence herein designated IREN, (depicted in Fig. 3), which encodes for a protein capable of binding to TRAF2, and the proteins encoded by this DNA sequences. The invention also concerns cDNA sequences of IREN isoforms IREN-10B and IREN-E (depicted in Figs. 4 and 5, respectively).

The DNA and the deduced amino acid sequences mentioned above do not appear in the 'GENEBANK' or 'PROTEIN BANK' data banks of DNA or amino acid sequences, they thus represent hitherto unknown sequences.

Within the scope of the present invention are also fragments of the above mentioned DNA sequences and DNA sequences capable of hybridization to those sequences or part of them, under moderately stringent conditions, provided they encode a biologically active protein or polypeptide capable of binding to at least the 225-501 amino acid sequence of TRAF2.

The present invention also concerns a DNA sequence which is degenerate as a result of the genetic code to the above mentioned DNA sequences and which encodes a biologically active protein or polypeptide capable of binding to at least the 225-501 amino acid sequence of TRAF2.

As regards TRAF2, it should be noted that several members of the TNF/NGF receptor family activate the transcription factor NF-κB by direct or indirect association with TRAF2, which is thus an adapter protein for these receptors and may thus also be considered as a modulator/mediator of the induction of NF-κB activation activity of these TNF/NGF receptors (see the scheme in Fig.2). Another receptor, the IL-1 receptor activates NF-κB independently of TRAF2. IREN analogs or muteins produced in accordance with the present invention (see

Examples) otherwise modul[illegible]NF-κB activation, when these analo[illegible]uteins are expressed in cells.

Thus, the present invention concerns the IREN protein, as well as the biologically active isoforms, analogs, fragments and derivatives thereof, and the isoforms, analogs, fragments and derivatives of the proteins encoded thereby. The preparation of such analogs, fragments and derivatives is by standard procedures (see for example, Sambrook et al., 1989) in which in the DNA encoding sequences, one or more codons may be deleted, added or substituted by another, to yield encoded analogs having at least a one amino acid residue change with respect to the native protein. Acceptable analogs are those which retain at least the capability of binding to TRAF2 with or without mediating any other binding or enzymatic activity, e.g. analogs which bind TRAF2 but do not signal, i.e. do not bind to a further downstream protein or other factor, or do not catalyze a signal-dependent reaction. In such a way analogs can be produced which have a so-called dominant-negative effect, namely, an analog, which is defective either in binding to TRAF2 or in subsequent signaling following such binding as, noted above. Such analogs can be used, for example, to inhibit the CD40, p55 TNF and p75 TNF (FAS/APO1 and other related receptor effects, as well as effected mediated by various receptor associated proteins (adapters) as noted above, by competing with the natural IREN proteins. Likewise, so-called dominant-positive analogs may be produced which would serve to enhance the TRAF2 effect. These would have the same or better TRAF2-binding properties and the same or better signaling properties than natural TRAF2-binding proteins. In an analogous fashion, biologically active fragments of the clones of the invention may be prepared as noted above with respect to the preparation of the analogs. Suitable fragments of the DNA sequences of the invention are those that encode a protein or polypeptide retaining the TRAF2 binding capability or which can mediate any other binding or enzymatic activity as noted above. Accordingly, fragments of the encoded proteins of the invention can be prepared which have a dominant-negative or a dominant-positive effect as noted above with respect to the analogs. Similarly, derivatives may be prepared by standard modifications of the side groups of one or more amino acid residues of the proteins, their analogs or fragments, or by conjugation of the proteins, their analogs or fragments, to another molecule e.g. an antibody, enzyme, receptor, etc., as are well known in the art.

Of the above DNA sequences of the invention which encode the TRAF2-binding protein IREN, biologically active isoforms, analogs, fragments or derivatives, there is also included, as an embodiment of the invention, DNA sequences capable of hybridizing with a

cDNA sequence derived from the coding region of a native TRAF-binding protein, in which such hybridization is performed under moderately stringent conditions, and which hybridizable DNA sequences encode a biologically active TRAF-binding protein. These hybridizable DNA sequences therefore include DNA sequences which have a relatively high homology to the native IREN cDNA sequence and as such represent TRAF-binding protein-like sequences which may be, for example, naturally-derived sequences encoding the various IREN isoforms, or naturally-occurring sequences encoding proteins belonging to a group of TRAF-binding protein-like sequences encoding IREN. Further, these sequences may also, for example, include non-naturally occurring, synthetically produced sequences, that are similar to the native IREN cDNA sequence but incorporate a number of desired modifications. Such synthetic sequences therefore include all of the possible sequences encoding analogs, fragments and derivatives of IREN, all of which have the activity of TRAF-binding proteins.

As used herein, stringency conditions are a function of the temperature used in the hybridization experiment, the molarity of the monovalent cations and the percentage of formamide in the hybridization solution. To determine the degree of stringency involved with any given set of conditions, one first uses the equation of Meinkoth et al. (1984) for determining the stability of hybrids of 100% identity expressed as melting temperature Tm of the DNA-DNA hybrid:

$$Tm = 81.5^0\, C + 16.6\ (LogM) + 0.41\ (\%GC) - 0.61\ (\%\ form) - 500/L$$

where M is the molarity of monovalent cations, %GC is the percentage of G and C nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. For each 1^0 C that the Tm is reduced from that calculated for a 100% identity hybrid, the amount of mismatch permitted is increased by about 1%. Thus, if the Tm used for any given hybridization experiment at the specified salt and formamide concentrations is 10^0 C below the Tm calculated for a 100% hybrid according to the equation of Meinkoth, hybridization will occur even if there is up to about 10% mismatch. Thus "highly stringent conditions" are those which provide a Tm which is not more than 10^0 C below the Tm that would exist for a perfect duplex with the target sequence, either as calculated by the above formula or as actually measured. "Moderately stringent conditions" are those which provide a Tm which is not more than 20^0C below the Tm that would exist for a perfect duplex with the target sequence, either as calculated by the above formula or as actually measured. Without limitation, examples of highly stringent (5-10^0 C below the calculated or measured Tm of the hybrid) and moderately stringent (15-20^0 C below the

calculated or measured Tm (of the hybrid) conditions use a wash solution of 2 X SSC (standard saline citrate) and 0.5% SDS (sodium dodecyl sulfate) at the appropriate temperature below the calculated Tm of the hybrid. The ultimate stringency of the conditions is primarily due to the washing conditions, particularly if the hybridization conditions used are those which allow less stable hybrids to form along with stable hybrids. The wash conditions at higher stringency then remove the less stable hybrids. A common hybridization condition that can be used with the highly stringent to moderately stringent wash conditions described above is hybridization in a solution of 6 X SSC (or 6 X SSPE (standard saline-phosphate-EDTA)), 5 X Denhardt's reagent, 0.5% SDS, 100 μg/ml denatured, fragmented salmon sperm DNA at a temperature approximately 20 to 25 C below the Tm. If mixed probes are used, it is preferable to use tetramethyl ammonium chloride (TMAC) instead of SSC (Ausubel, 1987, 1999).

To obtain the various above noted naturally occurring IREN-like sequences, standard procedures of screening and isolation of naturally-derived DNA or RNA samples from various tissues may be employed using the natural IREN cDNA or portion thereof as probe (see for example standard procedures set forth in Sambrook et al., 1989).

Likewise, to prepare the above noted various synthetic TRAF-binding protein-like sequences encoding analogs, fragments or derivatives of IREN, a number of standard procedures may be used as are detailed herein below concerning the preparation of such analogs, fragments and derivatives.

A polypeptide or protein "substantially corresponding" to IREN includes not only IREN itself but also polypeptides or proteins that are analogs of IREN.

Analogs that substantially correspond to IREN are those polypeptides in which one or more amino acid of IREN's amino acid sequence has been replaced with another amino acid, deleted and/or inserted, provided that the resulting protein exhibits substantially the same or higher biological activity as IREN.

In order to substantially correspond to IREN, the changes in the sequence of the proteins, such as isoforms are generally relatively minor. Although the number of changes may be more than ten, preferably there are no more than ten changes, more preferably no more than five, and most preferably no more than three such changes. While any technique can be used to find potentially biologically active proteins, which substantially correspond to IREN, one such technique is the use of conventional mutagenesis techniques on the DNA encoding

the protein, resulting in a few modifications. The proteins expressed by such clones can then be screened for their ability to bind to TRAF proteins (e.g. TRAF2) and to modulate TRAF protein (e.g. TRAF2) activity in modulation/mediation of the intracellular pathways noted above.

"Conservative" changes are those changes which would not be expected to change the activity of the protein and are usually the first to be screened as these would not be expected to substantially change the size, charge or configuration of the protein and thus would not be expected to change the biological properties thereof.

Conservative substitutions of IREN include an analog wherein at least one amino acid residue in the polypeptide has been conservatively replaced by a different amino acid. Such substitutions preferably are made in accordance with the following list as presented in Table IA, which substitutions may be determined by routine experimentation to provide modified structural and functional properties of a synthesized polypeptide molecule while maintaining the biological activity characteristic of IREN.

Table IA

Original Residue	Exemplary Substitution
Ala	Gly;Ser
Arg	Lys
Asn	Gln;His
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
Gly	Ala;Pro
His	Asn;Gln
Ile	Leu;Val
Leu	Ile;Val
Lys	Arg;Gln;Glu
Met	Leu;Tyr;Ile
Phe	Met;Leu;Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp;Phe
Val	Ile;Leu

Alternatively, another group of substitutions of IREN are those in which at least one amino acid residue in the polypeptide has been removed and a different residue inserted in its place according to the following Table IB. The types of substitutions which may be made in the polypeptide may be based on analysis of the frequencies of amino acid changes between a homologous protein of different species, such as those presented in Table 1-2 of Schulz et al., G.E., Principles of Protein Structure Springer-Verlag, New York, NY, 1798, and Figs. 3-9 of

Creighton, *T.E.*, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, CA 1983. Based on such an analysis, alternative conservative substitutions are defined herein as exchanges within one of the following five groups:

TABLE IB

1. Small aliphatic, nonpolar or slightly polar residues: Ala, Ser, Thr (Pro, Gly);
2. Polar negatively charged residues and their amides: Asp, Asn, Glu, Gln;
3. Polar, positively charged residues: His, Arg, Lys;
4. Large aliphatic nonpolar residues: Met, Leu, Ile, Val (Cys); and
5. Large aromatic residues: Phe, Tyr, Trp.

The three amino acid residues in parentheses above have special roles in protein architecture. Gly is the only residue lacking any side chain and thus imparts flexibility to the chain. This however tends to promote the formation of secondary structures other than α-helical. Pro, because of its unusual geometry, tightly constrains the chain and generally tends to promote ß-turn-like structures, although in some cases Cys can be capable of participating in disulfide bond formation, which is important in protein folding. Note that Schulz *et al., supra,* would merge Groups 1 and 2, above. Note also that Tyr, because of its hydrogen bonding potential, has significant kinship with Ser, and Thr, etc.

Conservative amino acid substitutions according to the present invention, e.g., as presented above, are known in the art and would be expected to maintain biological and structural properties of the polypeptide after amino acid substitution. Most deletions and substitutions according to the present invention are those which do not produce radical changes in the characteristics of the protein or polypeptide molecule. "Characteristics" is defined in a non-inclusive manner to define both changes in secondary structure, e.g. α-helix or ß-sheet, as well as changes in biological activity, e.g., binding to TRAF proteins and/or mediation of TRAF proteins' effect on cell death.

Examples of production of amino acid substitutions in proteins which can be used for obtaining analogs of IRENs for use in the present invention include any known method steps, such as presented in U.S. patent RE 33,653, 4,959,314, 4,588,585 and 4,737,462, to Mark et al.; 5,116,943 to Koths et al., 4,965,195 to Namen et al.; 4,879,111 to Chong et al.; and

5,017,691 to Lee et al.; a[illegible]ysine substituted proteins present i U.S. patent No. 4,904,584 (Shaw et al.).

Besides conservative substitutions discussed above which would not significantly change the activity of IREN, either conservative substitutions or less conservative and more random changes, which lead to an increase in biological activity of the analogs of IRENs, are intended to be within the scope of the invention.

When the exact effect of the substitution or deletion is to be confirmed, one skilled in the art will appreciate that the effect of the substitution(s), deletion(s), etc., will be evaluated by routine binding and cell death assays. Screening using such a standard test does not involve undue experimentation.

At the genetic level, these analogs are generally prepared by site-directed mutagenesis of nucleotides in the DNA encoding the IREN, thereby producing DNA encoding the analog, and thereafter synthesizing the DNA and expressing the polypeptide in recombinant cell culture. The analogs typically exhibit the same or increased qualitative biological activity as the naturally occurring protein, Ausubel *et al.*, Current Protocols in Molecular Biology, Greene Publications *and Wiley Interscience, New York, NY*, 1987-1995; Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989.

Preparation of an IREN mutein in accordance herewith, or an alternative nucleotide sequence encoding the same polypeptide but differing from the natural sequence due to changes permitted by the known degeneracy of the genetic code, can be achieved by site-specific mutagenesis of DNA that encodes an earlier prepared analog or a native version of an IREN. Site-specific mutagenesis allows the production of analogs through the use of specific oligonucleotide sequences that encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 20 to 25 nucleotides in length is preferred, with about 5 to 10 complementing nucleotides on each side of the sequence being altered. In general, the technique of site-specific mutagenesis is well known in the art, as exemplified by publications such as Adelman *et al., DNA* 2:183 (1983), the disclosure of which is incorporated herein by reference.

As will be appreciated, the site-specific mutagenesis technique typically employs a phage vector that exists in both a single-stranded and double-stranded form. Typical vectors

useful in site-directed mutagenesis include vectors such as the M13 phage, for example, as disclosed by Messing *et al., Third Cleveland Symposium on Macromolecules and Recombinant DNA*, Editor A. Walton, Elsevier, Amsterdam (1981), the disclosure of which is incorporated herein by reference. These phages are readily available commercially and their use is generally well known to those skilled in the art. Alternatively, plasmid vectors that contain a single-stranded phage origin of replication (Veira *et al., Meth. Enzymol.* 153:3, 1987) may be employed to obtain single-stranded DNA.

In general, site-directed mutagenesis in accordance herewith is performed by first obtaining a single-stranded vector that includes within its sequence a DNA sequence that encodes the relevant polypeptide. An oligonucleotide primer bearing the desired mutated sequence is prepared synthetically by automated DNA/oligonucleotide synthesis. This primer is then annealed with the single-stranded protein-sequence-containing vector, and subjected to DNA-polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, to complete the synthesis of the mutation-bearing strand. Thus, a mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as *E. coli* JM101 cells, and clones are selected that include recombinant vectors bearing the mutated sequence arrangement.

After such a clone is selected, the mutated IREN sequence may be removed and placed in an appropriate vector, generally a transfer or expression vector of the type that may be employed for transfection of an appropriate host.

Accordingly, a gene or nucleic acid coding for an IREN protein can also be detected, obtained and/or modified, *in vitro*, *in situ* and/or *in vivo*, by the use of known DNA or RNA amplification techniques, such as PCR and chemical oligonucleotide synthesis. PCR allows for the amplification (increase in number) of specific DNA sequences by repeated DNA polymerase reactions. This reaction can be used as a replacement for cloning; all that is required is knowledge of the nucleic acid sequence. In order to carry out PCR, primers are designed which are complementary to the sequence of interest. The primers are then generated by automated DNA synthesis. Because primers can be designed to hybridize to any part of the gene, conditions can be created such that mismatches in complementary base pairing can be tolerated. Amplification of these mismatched regions can lead to the synthesis of a mutagenized product resulting in the generation of a peptide with new properties (i.e., site directed mutagenesis). See also, e.g., Ausubel, *supra*, Ch. 16. Also, by coupling complementary DNA (cDNA) synthesis, using reverse transcriptase, with PCR, RNA can be

used as the starting materi r the synthesis of the extracellula main of a prolactin receptor without cloning.

Furthermore, PCR primers can be designed to incorporate new restriction sites or other features such as termination codons at the ends of the gene segment to be amplified. This placement of restriction sites at the 5' and 3' ends of the amplified gene sequence allows for gene segments encoding an IREN protein or a fragment thereof to be custom designed for ligation other sequences and/or cloning sites in vectors.

PCR and other methods of amplification of RNA and/or DNA are well known in the art and can be used according to the present invention without undue experimentation, based on the teaching and guidance presented herein. Known methods of DNA or RNA amplification include, but are not limited to polymerase chain reaction (PCR) and related amplification processes (see, e.g., U.S. patent Nos. 4,683,195, 4,683,202, 4,800,159, 4,965,188, to Mullis *et al.*; 4,795,699 and 4,921,794 to Tabor *et al.*; 5,142,033 to Innis; 5,122,464 to Wilson *et al.*; 5,091,310 to Innis; 5,066,584 to Gyllensten *et al.*; 4,889,818 to Gelfand *et al.*; 4,994,370 to Silver *et al.*; 4,766,067 to Biswas; 4,656,134 to Ringold; and Innis *et al.*, eds, *PCR Protocols: A Guide to Method and Applications*) and RNA mediated amplification which uses anti-sense RNA to the target sequence as a template for double stranded DNA synthesis (U.S. patent No. 5,130,238 to Malek *et al.*, with the trade name NASBA); and immuno-PCR which combines the use of DNA amplification with antibody labeling (Ruzicka *et al.*, *Science* 260:487 (1993); Sano *et al.*, *Science* 258:120 (1992); Sano *et al.*, *Biotechniques* 9:1378 (1991)), the entire contents of which patents and reference are entirely incorporated herein by reference.

In an analogous fashion, biologically active fragments of IREN or its isoforms may be prepared as noted above with respect to the analogs of TRAF-binding proteins. Suitable fragments of TRAF-binding proteins are those which retain the TRAF-binding protein capability and which can mediate the biological activity of TRAF proteins or other proteins associated with TRAF proteins directly or indirectly. Accordingly, IREN fragments can be prepared which have a dominant-negative or a dominant-positive effect as noted above with respect to the analogs. It should be noted that these fragments represent a special class of the analogs of the invention, namely, they are defined portions of IREN derived from the full IREN sequence or its isoforms, each such portion or fragment having any of the above-noted desired activities. Such fragment may be, e.g., a peptide.

Similarly, derivatives may be prepared by standard modifications of the side groups of one or more amino acid residues of IREN, its analogs or fragments, or by conjugation of the IREN, its analogs or fragments, to another molecule e.g. an antibody, enzyme, receptor, etc., as are well known in the art. Accordingly, "derivatives" as used herein covers derivatives which may be prepared from the functional groups which occur as side chains on the residues or the N- or C-terminal groups, by means known in the art, and are included in the invention. Derivatives may have chemical moieties such as carbohydrate or phosphate residues, provided such a fraction has the same or higher biological activity than IREN proteins.

For example, derivatives may include aliphatic esters of the carboxyl groups, amides of the carboxyl groups by reaction with ammonia or with primary or secondary amines, N-acyl derivatives or free amino groups of the amino acid residues formed with acyl moieties (e.g., alkanoyl or carbocyclic aroyl groups) or O-acyl derivatives of free hydroxyl group (for example that of seryl or threonyl residues) formed with acyl moieties.

The term "derivatives" is intended to include only those derivatives that do not change one amino acid to another of the twenty commonly occurring natural amino acids.

An IREN protein is a protein or polypeptide, i.e. a sequence of amino acid residues. A polypeptide consisting of a larger sequence which includes the entire sequence of an IREN protein, in accordance with the definitions herein, is intended to be included within the scope of such a polypeptide as long as the additions do not affect the basic and novel characteristics of the invention, i.e., if they either retain or increase the biological activity of IREN or can be cleaved to leave a protein or polypeptide having the biological activity of IREN. Thus, for example, the present invention is intended to include fusion proteins of IREN with other amino acids or peptides.

As mentioned above, it should be understood that the above IREN, isoforms, fragments, derivatives, muteins etc. of the invention are any proteins which may bind and/or mediate/modulate the activity of any TRAF protein intracellularly. In particular, examples are those proteins which can modulate or mediate the TRAF2-associated intracellular signaling activity, as mentioned above, especially as concerns TRAF2's involvement in modulating NF-κB activity, in particular, following the interaction between TRAF2 and various members of the TNF/NGF receptor family and/or their associated adapter proteins as detailed above and below. IREN according to the invention and its various isoforms analogs, fragments, etc. (see Examples) which appear to bind TRAF2 very specifically and to have an action in modulating

NF-κB activity, with IREN inant-negative analogs/muteins mo ing this activity, do so.

All the above mentioned modifications are in the scope of the invention provided they preserved the ability of the encoded proteins or polypeptides or their analogs and derivatives thereof, to bind at least the 225-501 amino acid sequence of TRAF2.

All the proteins and polypeptides of the invention by virtue of their capability to bind to TRAF2, are considered as mediators or modulators of TRAF2 signaling. As such, said molecules of the invention have a role in, for example, the signaling process in which the binding of TRAF2 ligand to CD30, CD40, lymphotoxin beta (LT-β) receptor, p55 or p75 TNF receptors, as well as the other receptors and adaptor proteins noted herein above, leads to activation of the transcription factor NF-κB. Particularly interesting is protein IREN and its isoforms of the invention.

The new clones, proteins, their analogs, fragments and derivatives have a number of possible uses, for example:

(i) They may be used to modulate NFκB activity, the function of TRAF2 and the receptors to which they bind, in situations where a modulation of function is desired such as in anti-tumor or immuno-stimulatory applications where the TRAF2- induced effects are desired. In this case the proteins of the invention, their analogs, fragments or derivatives, which modulate the TRAF2 or receptors effects, may be introduced to the cells by standard procedures known <u>per se</u>. For example, as the proteins encoded by the DNA clones of the invention are intracellular and they should be introduced only into the cells where the TRAF2 effect is desired, a system for specific introduction of these proteins into the cells is necessary. One way of doing this is by creating a recombinant animal virus e.g. one derived from Vaccinia, to the DNA of which the following two genes will be introduced: the gene encoding a ligand that binds to cell surface proteins specifically expressed by the cells e.g. ones such as the AIDs (HIV) virus gp120 protein which binds specifically to some cells (CD4 lymphocytes and related leukemias) or any other ligand that binds specifically to cells carrying a receptor that binds TRAF2, such that the recombinant virus vector will be capable of binding such cells; and the gene encoding the proteins of the invention. Thus, expression of the cell-surface-binding protein on the surface of the virus will target the virus specifically to the tumor cell or other receptor- carrying cell, following which the proteins encoding sequences will be introduced into the cells via the virus, and once expressed in the cells will result in

enhancement of the receptor or TRAF2 effect leading to a desired immuno-stimulatory effect in these cells. Construction of such recombinant animal virus is by standard procedures (see for example, Sambrook et al., 1989). Another possibility is to introduce the sequences of the encoded proteins in the form of oligonucleotides, which can be absorbed by the cells and expressed therein.

(ii) They may be used to modulate the NFκB activity, the effects of TRAF2 or of the receptor that binds it, e.g. in cases such as tissue damage as in AIDS, septic shock or graft-vs.-host rejection, in which it is desired to block the induced intracellular signaling. In this situation it is possible, for example, to introduce into the cells, by standard procedures, oligonucleotides having the anti-sense coding sequence for the proteins of the invention, which would effectively block the translation of mRNAs encoding the proteins and thereby block their expression and lead to the inhibition of the undesired effect. Alternatively, other oligonucleotides may be used; oligonucleotides that preserved their ability to bind to TRAF2 in a way that interferes with the binding of other molecules to this protein, while at the same time do not mediate any activation or modulation of this molecule. Having these characteristics, said molecules can disrupt the interaction of TRAF2 with its natural ligand, therefor acting as inhibitors capable of abolishing effects mediated by TRAF2, such as NF-κB activation, for example. Such oligonucleotides may be introduced into the cells using the above recombinant virus approach, the second sequence carried by the virus being the oligonucleotide sequence.

Another possibility is to use antibodies specific for the proteins of the invention to inhibit their intracellular signaling activity.

Yet another way of inhibiting the undesired effect is by the recently developed ribozyme approach. Ribozymes are catalytic RNA molecules that specifically cleave RNAs. Ribozymes may be engineered to cleave target RNAs of choice, e.g. the mRNAs encoding the proteins of the invention. Such ribozymes would have a sequence specific for the mRNA of the proteins and would be capable of interacting therewith (complementary binding) followed by cleavage of the mRNA, resulting in a decrease (or complete loss) in the expression of the proteins, the level of decreased expression being dependent upon the level of ribozyme expression in the target cell. To introduce ribozymes into the cells of choice (e.g. those carrying the IREN proteins) any suitable vector may be used, e.g. plasmid, animal virus (retrovirus) vectors, that are usually used for this purpose (see also (i) above, where the virus

has, as second sequence, a A encoding the ribozyme sequence hoice). (For reviews, methods etc. concerning ribozymes see Chen et al., 1992; Zhao and Pick, 1993).

(iii) They may be used to isolate, identify and clone other proteins which are capable of binding to them, e.g. other proteins involved in the intracellular signaling process that are downstream of TRAF2. For example, the DNA sequences encoding the proteins of the invention may be used in the yeast two-hybrid system in which the encoded proteins will be used as "bait" to isolate, clone and identify from cDNA or genomic DNA libraries other sequences ("preys") encoding proteins which can bind to the cloned proteins. In the same way, it may also be determined whether the proteins of the present invention can bind to other cellular proteins, e.g. other receptors of the TNF/NGF superfamily of receptors.

(iv) The encoded proteins, their analogs, fragments or derivatives may also be used to isolate, identify and clone other proteins of the same class i.e. those binding to TRAF2 or to functionally related proteins, and involved in the intracellular signaling process. In this application the above noted yeast two-hybrid system may be used, or there may be used a recently developed system employing non-stringent Southern hybridization followed by PCR cloning (Wilks et al., 1989).

(v) Yet another approach to utilize the encoded proteins of the invention, their isoforms analogs, fragments or derivatives is to use them in methods of affinity chromatography to isolate and identify other proteins or factors to which they are capable of binding, e.g., proteins related to TRAF2 or other proteins or factors involved in the intracellular signaling process. In this application, the protein, its isoforms analogs, fragments or derivatives of the present invention, may be individually attached to affinity chromatography matrices and then brought into contact with cell extracts or isolated proteins or factors suspected of being involved in the intracellular signaling process. Following the affinity chromatography procedure, the other proteins or factors which bind to the proteins, their analogs, fragments or derivatives of the invention, can be eluted, isolated and characterized.

(vi) As noted above, the proteins, their analogs, fragments or derivatives of the invention may also be used as immunogens (antigens) to produce specific antibodies thereto. These antibodies may also be used for the purposes of purification of the protein of the invention either from cell extracts or from transformed cell lines producing them, their analogs or fragments. Further, these antibodies may be used for diagnostic purposes for identifying disorders related to abnormal functioning of the receptor system in which they

function, e.g., overactive or under active TRAF2- induced cellular effects. Thus, should such disorders be related to a malfunctioning intracellular signaling system involving the proteins of the invention, such antibodies would serve as an important diagnostic tool. The term "antibody" is meant to include polyclonal antibodies, monoclonal antibodies (mAbs), chimeric antibodies, anti-idiotypic (anti-Id) antibodies to antibodies that can be labeled in soluble or bound form, as well as fragments thereof, such as, for example, Fab and $F(ab')_2$ - fragments lacking the Fc fragment of intact antibody, which are capable of binding antigen.

(vii) The antibodies, including fragments of antibodies, useful in the present invention may be used to quantitatively or qualitatively detect the clones of the invention in a sample, or to detect presence of cells which express the clones of the present invention. This can be accomplished by immunofluorescence techniques employing a fluorescently labeled antibody coupled with light microscopic, flow cytometric, or fluorometric detection.

The antibodies (or fragments thereof) useful in the present invention may be employed histologically, as in immunofluorescence or immunoelectron microscopy, for *in situ* detection of the clones of the present invention. *In situ* detection may be accomplished by removing a histological specimen from a patient, and providing the labeled antibody of the present invention to such a specimen. The antibody (or fragment) is preferably provided by applying or by overlaying the labeled antibody (or fragment) to a biological sample. Through the use of such a procedure, it is possible to determine not only the presence of the clones, but also its distribution on the examined tissue. Using the present invention, those of ordinary skill will readily perceive that any of wide variety of histological methods (such as staining procedures) can be modified in order to achieve such *in situ* detection.

Such assays for the clones of the present invention typically comprise incubating a biological sample, such as a biological fluid, a tissue extract, freshly harvested cells such as lymphocytes or leukocytes, or cells which have been incubated in tissue culture, in the presence of a labeled antibody capably of identifying the encoded proteins, and detecting the antibody by any of a number of techniques well known in the art.

(viii) The encoded proteins of the invention may also be used as indirect modulators of a number of other proteins by virtue of their capability of binding to other intracellular proteins, which other intracellular proteins directly bind yet other intracellular proteins or an intracellular domain of a transmembrane protein.

For the purposes of ulating these other intracellular pro or the intracellular domains of transmembranal proteins, the proteins of the invention may be introduced into cells in a number of ways as mentioned hereinabove in (ii).

It should also be noted that the isolation, identification and characterization of the proteins of the invention might be performed using any of the well-known standard screening procedures. For example, one of these screening procedures, the yeast two-hybrid procedure which was used to identify the proteins of the invention. Likewise other procedures may be employed such as affinity chromatography, DNA hybridization procedures, etc. as are well known in the art, to isolate, identify and characterize the proteins of the invention or to isolate, identify and characterize additional proteins, factors, receptors, etc. which are capable of binding to the proteins of the invention.

Moreover, the proteins found to bind to the proteins of the invention may themselves be employed, in an analogous fashion to the way in which the proteins of the invention were used as noted above and below, to isolate, identify and characterize other proteins, factors, etc. which are capable of binding to the proteins of the invention-binding proteins and which may represent factors involved further downstream in the associated signaling process, or which may have signaling activities of their and hence would represent proteins involved in a distinct signaling process.

The DNA sequences and the encoded proteins of the invention may be produced by any standard recombinant DNA procedure (see for example, Sambrook, et al., 1989) in which suitable eukaryotic or prokaryotic host cells are transformed by appropriate eukaryotic or prokaryotic vectors containing the sequences encoding for the proteins. Accordingly, the present invention also concerns such expression vectors and transformed hosts for the production of the proteins of the invention. As mentioned above, these proteins also include their biologically active analogs, fragments and derivatives, and thus the vectors encoding them also include vectors encoding analogs and fragments of these proteins, and the transformed hosts include those producing such analogs and fragments. The derivatives of these proteins are the derivatives produced by standard modification of the proteins or their analogs or fragments, produced by the transformed hosts.

The present invention also relates to pharmaceutical compositions for modulation of the effects mediated by TRAF2. The pharmaceutical compositions comprising, as an active ingredient, any one or more of the following: (i) one or more of the DNA sequences of the invention, or parts of them, subcloned into an appropriate expression vector; (ii) a protein

according to the invention, its biologically active fragments, analogs, derivatives or a mixture thereof; (iii) a recombinant animal virus vector encoding for a protein according to the invention, its biologically active fragments, analogs or derivatives.

The pharmaceutical compositions are applied according to the disease to be treated and in amounts beneficial to the patent, depending on body weight and other considerations, as determined by the physician.

As noted above, one of the specific embodiments of the TRAF-binding proteins of the present invention is the TRAF2-binding protein IREN. Based on the findings in accordance with the present invention that IREN binds specifically to TRAF2 and as such is a mediator/modulator of TRAF2 and can thus mediate/modulate TRAF2's activity in NF-κB activation and hence its possible role in cell survival pathways in ways that TRAF2 functions independently or in conjunction with other proteins (e.g. p55 TNF and p75 TNF receptors, FAS/APO1 receptor, MORT-1, RIP and TRADD) it is of importance to design drugs which may enhance or inhibit the TRAF2-IREN interaction, as desired. For example, when it is desired to modulate the cell cytotoxicity induced by TNF it would be desired to modulate NF-κB induction, by modulating the TRAF2-IREN interaction or by modulating TRAF2 and/or IREN specifically. Likewise, for example, when it is desired to modulate the cell cytotoxicity induced by TNF it would be desired to modulate NF-κB induction by modulating the TRAF2-IREN interaction or by modulating TRAF2- and/or IREN specific NF-κB modulation. There are many diseases in which such drugs can be of great help. Amongst others, (see above discussion as well) acute hepatitis in which the acute damage to the liver seems to reflect FAS/APO1 receptor-mediated death of the liver cells following induction by the Fas ligand; autoimmune-induced cell death such as the death of the ß Langerhans cells of the pancreas, that results in diabetes; the death of cells in graft rejection (e.g., kidney, heart and liver); the death of oligodendrocytes in the brain in multiple sclerosis; and AIDS-inhibited T cell suicide which causes proliferation of the AIDS virus and hence the AIDS disease.

It is possible that IREN or one or more of its possible biologically active isoforms, analogs or fragments may serve as "natural" inhibitors of IREN itself or of the IREN-TRAF2 interaction, and as such serve as modulators of NF-κB activation. Such modulators may thus be employed as the specific modulators noted above, for example, those modulators to be used when it is desired to modulate the cell cytotoxic effects of TNF. In fact, as exemplified herein below, various IREN analogs and muteins have been isolated in accordance with the

present invention, which are capable of modulating the induction of NF-κB activation mediated by NIK, NEMO, IKK-1 or fragments thereof. And also as mediated by bacterial endotoxin (LPS), phorbol myristate acetate, and the HTLV-1 protein TAX. Likewise, other substances such as peptides, organic compounds, antibodies, etc. may also be screened to obtain specific drugs, which are capable of inhibiting the TRAF2-IREN interaction or the activity of IREN.

In a similar fashion, when it is desired to modulate the NF-κB activation in various situations as noted above it is possible, for example, to modulate the amount of IREN and/or TRAF2 in cells by various standard methods noted herein above (e.g. introducing DNA encoding IREN and/or TRAF2 into cells to modulate expression, or preparing suitable formulations containing IREN and/or TRAF2 for direct introduction into cells, or any other way known to those of skill in the art). Likewise, other substances such as peptides, organic compounds, etc. may also be screened to obtain specific drugs, which are capable of enhancing the activity of IREN or of enhancing the TRAF2- IREN interaction.

A non-limiting example of how peptide modulators of the IREN-TRAF2 interaction would be designed and screened is based on previous studies on peptide inhibitors of ICE or ICE-like proteases, the substrate specificity of ICE and strategies for epitope analysis using peptide synthesis. The minimum requirement for efficient cleavage of a peptide by ICE was found to involve four amino acids to the left of the cleavage site with a strong preference for aspartic acid in the P_1 position and with methylamine being sufficient to the right of the P_1 position (Sleath et al., 1990; Howard et al., 1991; Thornberry et al., 1992). Furthermore, the fluorogenic substrate peptide (a tetrapeptide), acetyl-Asp-Glu-Val-Asp-a-(4-methyl-coumaryl-7-amide) abbreviated Ac-DEVD-AMC, corresponds to a sequence in poly (ADP-ribose) polymerase (PARP) found to be cleaved in cells shortly after FAS-R stimulation, as well as other apoptopic processes (Kaufmann, 1989; Kaufmann et al., 1993; Lazebnik et al., 1994), and is cleaved effectively by CPP32 (a member of the CED3/ICE protease family) and MACH proteases.

As Asp in the P_1 position of the substrate appears to be important, tetrapeptides having Asp as the fourth amino acid residue and various combinations of amino acids in the first three residue positions can be rapidly screened for binding to the active site of the proteases using, for example, the method developed by Geysen (Geysen, 1985; Geysen et al., 1987) where a large number of peptides on solid supports were screened for specific interactions with antibodies. The binding of MACH proteases to specific peptides can be detected by a

variety of well known detection methods within the skill of those in the art, such as radiolabeling, etc. This method of Geysen's was shown to be capable of testing at least 4000 peptides each working day.

In a similar way the exact binding region or region of homology which determines the interaction between TRAF2 and IREN (or any other TRAF protein and TRAF-binding protein) can be elucidated and then peptides may be screened which can serve to block this interaction. e.g. peptides synthesized having a sequence similar to that of the binding region or complementary thereto which can compete with natural IREN (or TRAF-binding protein) for binding to TRAF2 (or TRAF).

Since it may be advantageous to design peptide inhibitors that selectively inhibit TRAF2-IREN (or TRAF-TRAF binding protein) interactions without interfering with physiological cell death processes in which other members of the intracellular signaling pathway are involved, e.g. MACH proteases of the cell death pathway, which are members of the CED3/ICE family of proteases, the pool of peptides binding to TRAF2 (or TRAF) or IREN (or TRAF-binding proteins) in an assay such as the one described above can be further synthesized as a fluorogenic substrate peptide to test for selective binding to such other proteins to select only those specific for TRAF2/ IREN (or TRAF/TRAF-binding protein). Peptides, which are determined to be specific for, for example, TRAF2/IREN, can then be modified to enhance cell permeability and inhibit the activity of TRAF2 and/or IREN either reversibly or irreversibly. Thornberry et al. (1994) reported that a tetrapeptide (acyloxy) methyl ketone Ac-Tyr-Val-Ala-Asp-CH_2OC (O)-[2,6-$(CF_3)_2$] Ph was a potent inactivator of ICE. Similarly, Milligan et al. (1995) reported that tetrapeptide inhibitors having a chloromethylketone (irreversibly) or aldehyde (reversibly) groups inhibited ICE. In addition, a benzyloxycarboxyl-Asp-CH_2OC (O) -2,6-dichlorobenzene (DCB) was shown to inhibit ICE (Mashima et al., 1995). Accordingly, in an analogous way, tetrapeptides that selectively bind to, for example, TRAF2 or IREN, can be modified with, for example, an aldehyde group, chloromethylketone,(acyloxy) methyl ketone or a CH_2OC (O)-DCB group to create a peptide inhibitor of TRAF2/IREN activity. Further, to improve permeability, peptides can be, for example, chemically modified or derivatized to enhance their permeability across the cell membrane and facilitate the transport of such peptides through the membrane and into the cytoplasm. Muranishi et.al. (1991) reported derivatizing thyrotropin-releasing hormone with lauric acid to form a lipophilic lauroyl derivative with good penetration characteristics across cell membranes. Zacharia et al. (1991) also reported the oxidation of methionine to sulfoxide

and the replacement of the peptide bond with its ketomethylene isoester ($COCH_2$) to facilitate transport of peptides through the cell membrane. These are just some of the known modifications and derivatives that are well within the skill of those in the art.

Furthermore, drug or peptide inhibitors, which are capable of inhibiting the activity of, for example, IREN by inhibiting the IREN-TRAF2 interaction and likewise, the interaction between TRAF proteins and TRAF-binding proteins can be conjugated or complexed with molecules that facilitate entry into the cell.

U.S. Patent 5,149,782 discloses conjugating a molecule to be transported across the cell membrane with a membrane blending agent such as fusogenic polypeptides, ion-channel forming polypeptides, other membrane polypeptides, and long chain fatty acids, e.g. myristic acid, palmitic acid. These membrane blending agents insert the molecular conjugates into the lipid bilayer of cellular membranes and facilitate their entry into the cytoplasm.

Low et al., U.S. Patent 5, 108,921, reviews available methods for transmembrane delivery of molecules such as, but not limited to, proteins and nucleic acids by the mechanism of receptor mediated endocytotic activity. These receptor systems include those recognizing galactose, mannose, mannose 6-phosphate, transferrin, asialoglycoprotein, transcobalamin (vitamin B_{12}), α-2 macroglobulins, insulin and other peptide growth factors such as epidermal growth factor (EGF). Low et al. teaches that nutrient receptors, such as receptors for biotin and folate, can be advantageously used to enhance transport across the cell membrane due to the location and multiplicity of biotin and folate receptors on the membrane surfaces of most cells and the associated receptor mediated transmembrane transport processes. Thus, a complex formed between a compound to be delivered into the cytoplasm and a ligand, such as biotin or folate, is contacted with a cell membrane bearing biotin or folate receptors to initiate the receptor mediated trans-membrane transport mechanism and thereby permit entry of the desired compound into the cell.

ICE is known to have the ability to tolerate liberal substitutions in the P_2 position and this tolerance to liberal substitutions was exploited to develop a potent and highly selective affinity label containing a biotin tag (Thornberry et al., 1994). Consequently, the P_2 position as well as possibly the N-terminus of the tetrapeptide inhibitor can be modified or derivatized, such as to with the addition of a biotin molecule, to enhance the permeability of these peptide inhibitors across the cell membrane.

In addition, it is known in the art that fusing a desired peptide sequence with a leader/signal peptide sequence to create a "chimeric peptide" will enable such a "chimeric peptide" to be transported across the cell membrane into the cytoplasm.

As will be appreciated by those of skill in the art of peptides, the peptide inhibitors of the TRAF-TRAF-binding protein interaction, for example, the TRAF2-IREN interaction according to the present invention is meant to include peptidomimetic drugs or inhibitors, which can also be rapidly screened for binding to, for example TRAF2/IREN to design perhaps more stable inhibitors.

It will also be appreciated that the same means for facilitating or enhancing the transport of peptide inhibitors across cell membranes as discussed above are also applicable to the TRAF-binding proteins, for example, IREN, its analogs, fragments or its isoforms themselves as well as other peptides and proteins which exert their effects intracellularly.

As regards the antibodies mentioned herein throughout, the term "antibody" is meant to include polyclonal antibodies, monoclonal antibodies (mAbs), chimeric antibodies, anti-idiotypic (anti-Id) antibodies to antibodies that can be labeled in soluble or bound form, as well as fragments thereof provided by any known technique, such as, but not limited to enzymatic cleavage, peptide synthesis or recombinant techniques.

Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of animals immunized with an antigen. A monoclonal antibody contains a substantially homogeneous population of antibodies specific to antigens, which populations contains substantially similar epitope binding sites. MAbs may be obtained by methods known to those skilled in the art. See, for example Kohler and Milstein, Nature, 256:495-497 (1975); U.S. Patent No. 4,376,110; Ausubel et al., eds., Harlow and Lane ANTIBODIES : A LABORATORY MANUAL, Cold Spring Harbor Laboratory (1988); and Colligan et al., eds., Current Protocols in Immunology, Greene Publishing Assoc. and Wiley Interscience N.Y., (1992-1996), the contents of which references are incorporated entirely herein by reference. Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, GILD and any subclass thereof. A hybridoma producing a mAb of the present invention may be cultivated *in vitro, in situ* or *in vivo*. Production of high titers of mAbs *in vivo* or *in situ* makes this the presently preferred method of production.

Chimeric antibodies are molecules of which different portions are derived from different animal species, such as those having the variable region derived from a murine mAb and a human immunoglobulin constant region. Chimeric antibodies are primarily used to

reduce immunogenicity in [illegible]ication and to increase yields in p[illegible]ction, for example, where murine mAbs have higher yields from hybridomas but higher immunogenicity in humans, such that human/murine chimeric mAbs are used. Chimeric antibodies and methods for their production are known in the art (Cabilly et al., *Proc. Natl. Acad. Sci. USA* 81:3273-3277 (1984); Morrison et al., *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984); Boulianne et al., *Nature* 312:643-646 (1984); Cabilly et al., European Patent Application 125023 (published November 14, 1984); Neuberger et al., *Nature* 314:268-270 (1985); Taniguchi et al., European Patent Application 171496 (published February 19, 1985); Morrison et al., European Patent Application 173494 (published March 5, 1986); Neuberger et al., PCT Application WO 8601533, (published March 13, 1986); Kudo et al., European Patent Application 184187 (published June 11, 1986); Sahagan et al., *J. Immunol.* 137:1066-1074 (1986); Robinson et al., International Patent Application No. WO8702671 (published May 7, 1987); Liu et al., *Proc. Natl. Acad. Sci USA* 84:3439-3443 (1987); Sun et al., *Proc. Natl. Acad. Sci USA* 84:214-218 (1987); Better et al., *Science* 240:1041-1043 (1988); and Harlow and Lane, ANTIBODIES: A LABORATORY MANUAL, supra. These references are entirely incorporated herein by reference.

An anti-idiotypic (anti-Id) antibody is an antibody which recognizes unique determinants generally associated with the antigen-binding site of an antibody. An Id antibody can be prepared by immunizing an animal of the same species and genetic type (e.g. mouse strain) as the source of the mAb to which an anti-Id is being prepared. The immunized animal will recognize and respond to the idiotypic determinants of the immunizing antibody by producing an antibody to these idiotypic determinants (the anti-Id antibody). See, for example, U.S. Patent No. 4,699,880, which is herein entirely incorporated by reference.

The anti-Id antibody may also be used as an "immunogen" to induce an immune response in yet another animal, producing a so-called anti-anti-Id antibody. The anti-anti-Id may be epitopically identical to the original mAb, which induced the anti-Id. Thus, by using antibodies to the idiotypic determinants of a mAb, it is possible to identify other clones expressing antibodies of identical specificity.

Accordingly, mAbs generated against IREN, its isoforms, analogs, fragments or derivatives of the present invention may be used to induce anti-Id antibodies in suitable animals, such as BALB/c mice. Spleen cells from such immunized mice are used to produce anti-Id hybridomas secreting anti-Id mAbs. Further, the anti-Id mAbs can be coupled to a carrier such as keyhole limpet hemocyanin (KLH) and used to immunize additional BALB/c

mice. Sera from these mice will contain anti-anti-Id antibodies that have the binding properties of the original mAb specific for an epitope of the above IREN protein, or analogs, fragments and derivatives thereof.

The anti-Id mAbs thus have their own idiotypic epitopes, or "idiotopes" structurally similar to the epitope being evaluated, such as GRB protein-a.

The term "antibody" is also meant to include both intact molecules as well as fragments thereof, such as, for example, Fab and F(ab')2, which are capable of binding antigen. Fab and F(ab')2 fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding than an intact antibody (Wahl et al., *J. Nucl. Med.* 24:316-325 (1983)).

It will be appreciated that Fab and F(ab')2 and other fragments of the antibodies useful in the present invention may be used for the detection and quantitation of the IREN protein according to the methods disclosed herein for intact antibody molecules. Such fragments are typically produced by proteolytic cleavage, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce F(ab')2 fragments).

An antibody is said to be "capable of binding" a molecule if it is capable of specifically reacting with the molecule to thereby bind the molecule to the antibody. The term "epitope" is meant to refer to that portion of any molecule capable of being bound by an antibody, which can also be recognized by that antibody. Epitopes or "antigenic determinants" usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and have specific three dimensional structural characteristics as well as specific charge characteristics.

An "antigen" is a molecule or a portion of a molecule capable of being bound by an antibody, which is additionally capable of inducing an animal to produce antibody capable of binding to an epitope of that antigen. An antigen may have one or more than one epitope. The specific reaction referred to above is meant to indicate that the antigen will react, in a highly selective manner, with its corresponding antibody and not with the multitude of other antibodies which may be evoked by other antigens.

The antibodies, including fragments of antibodies, useful in the present invention may be used to quantitatively or qualitatively detect the IREN proteins in a sample or to detect presence of cells that express the IREN proteins of the present invention. This can be accomplished by immunofluorescence techniques employing a fluorescently labeled antibody (see below) coupled with light microscopic, flow cytometric, or fluorometric detection.

The antibodies (or ments thereof) useful in the pre invention may be employed histologically, as in immunofluorescence or immunoelectron microscopy, for *in situ* detection of the IREN proteins of the present invention. *In situ* detection may be accomplished by removing a histological specimen from a patient, and providing the labeled antibody of the present invention to such a specimen. The antibody (or fragment) is preferably provided by applying or by overlaying the labeled antibody (or fragment) to a biological sample. Through the use of such a procedure, it is possible to determine not only the presence of the IREN proteins but also its distribution on the examined tissue. Using the present invention, those of ordinary skill will readily perceive that any of wide variety of histological methods (such as staining procedures) can be modified in order to achieve such *in situ* detection.

Such assays for the IREN proteins of the present invention typically comprises incubating a biological sample, such as a biological fluid, a tissue extract, freshly harvested cells such as lymphocytes or leukocytes, or cells which have been incubated in tissue culture, in the presence of a labeled antibody capable of identifying the IREN proteins, and detecting the antibody by any of a number of techniques well known in the art.

The biological sample may be treated with a solid phase support or carrier such as nitrocellulose, or other solid support or carrier which is capable of immobilizing cells, cell particles or soluble proteins. The support or carrier may then be washed with suitable buffers followed by treatment with a labeled antibody in accordance with the present invention, as noted above. The solid phase support or carrier may then be washed with the buffer a second time to remove unbound antibody. The amount of bound label on said solid support or carrier may then be detected by conventional means.

By "solid phase support", "solid phase carrier", "solid support", "solid carrier", "support" or "carrier" is intended any support or carrier capable of binding antigen or antibodies. Well-known supports or carriers, include glass, polystyrene, polypropylene, polyethylene, dextran, nylon amylases, natural and modified celluloses, polyacrylamides, gabbros and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. The support material may have virtually any possible structural configuration so long as the coupled molecule is capable of binding to an antigen or antibody. Thus, the support or carrier configuration may be spherical, as in a bead, cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc. Preferred supports or

carriers include polystyrene beads. Those skilled in the art will know may other suitable carriers for binding antibody or antigen, or will be able to ascertain the same by use of routine experimentation.

The binding activity of a given lot of antibody, of the invention as noted above, may be determined according to well-known methods. Those skilled in the art will be able to determine operative and optimal assay conditions for each determination by employing routine experimentation.

Other such steps as washing, stirring, shaking, filtering and the like may be added to the assays as is customary or necessary for the particular situation.

One of the ways in which an antibody in accordance with the present invention can be labeled is by linking the same to an enzyme and used in an enzyme immunoassay (EIA). This enzyme, in turn, when later exposed to an appropriate substrate, will react with the substrate in such a manner as to produce a chemical moiety which can be detected, for example, by spectrophotometric, fluorometric or by visual means. Enzymes which can be used to detectably label the antibody include, but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomeras, yeast alcohol dehydrogenase, alpha-glycerophosphate dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholin-esterase. The detection can be accomplished by colorimetric methods which employ a chromogenic substrate for the enzyme. Detection may also be accomplished by visual comparison of the extent of enzymatic reaction of a substrate in comparison with similarly prepared standards.

Detection may be accomplished using any of a variety of other immunoassays. For example, by radioactive labeling the antibodies or antibody fragments, it is possible to detect R-PTPase through the use of a radioimmunoassay (RIA). A good description of RIA may be found in Laboratory Techniques and Biochemistry in Molecular Biology, by Work, T.S. et al., North Holland Publishing Company, NY (1978) with particular reference to the chapter entitled "An Introduction to Radioimmune Assay and Related Techniques" by Chard, T., incorporated by reference herein. The radioactive isotope can be detected by such means as the use of a g counter or a scintillation counter or by autoradiography.

It is also possible to label an antibody in accordance with the present invention with a fluorescent compound. When the fluorescently labeled antibody is exposed to light of the proper wavelength, its presence can be then detected due to fluorescence. Among the most

commonly used fluorescent labeling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrine, pycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

The antibody can also be detectably labeled using fluorescence emitting metals such as ^{152}E, or others of the lanthanide series. These metals can be attached to the antibody using such metal chelating groups as diethylenetriamine pentaacetic acid (ETPA).

The antibody can also be detectably labeled by coupling it to a chemiluminescent compound. The presence of the chemiluminescent-tagged antibody is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

Likewise, a bioluminescent compound may be used to label the antibody of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

An antibody molecule of the present invention may be adapted for utilization in an immunometric assay, also known as a "two-site" or "sandwich" assay. In a typical immunometric assay, a quantity of unlabeled antibody (or fragment of antibody) is bound to a solid support or carrier and a quantity of detectably labeled soluble antibody is added to permit detection and/or quantitation of the ternary complex formed between solid-phase antibody, antigen, and labeled antibody.

Typical, and preferred, immunometric assays include "forward" assays in which the antibody bound to the solid phase is first contacted with the sample being tested to extract the antigen from the sample by formation of a binary solid phase antibody-antigen complex. After a suitable incubation period, the solid support or carrier is washed to remove the residue of the fluid sample, including unreacted antigen, if any, and then contacted with the solution containing an unknown quantity of labeled antibody (which functions as a "reporter molecule"). After a second incubation period to permit the labeled antibody to complex with the antigen bound to the solid support or carrier through the unlabeled antibody, the solid support or carrier is washed a second time to remove the unreacted labeled antibody.

In another type of "sandwich" assay, which may also be useful with the antigens of the present invention, the so-called "simultaneous" and "reverse" assays are used. A simultaneous

assay involves a single incubation step as the antibody bound to the solid support or carrier and labeled antibody are both added to the sample being tested at the same time. After the incubation is completed, the solid support or carrier is washed to remove the residue of fluid sample and uncomplexed labeled antibody. The presence of labeled antibody associated with the solid support or carrier is then determined, as it would be in a conventional "forward" sandwich assay.

In the "reverse" assay, stepwise addition first of a solution of labeled antibody to the fluid sample followed by the addition of unlabeled antibody bound to a solid support or carrier after a suitable incubation period is utilized. After a second incubation, the solid phase is washed in conventional fashion to free it of the residue of the sample being tested and the solution of unreacted labeled antibody. The determination of labeled antibody associated with a solid support or carrier is then determined as in the "simultaneous" and "forward" assays.

As mentioned above, the present invention also relates to pharmaceutical compositions comprising recombinant animal virus vectors encoding the IREN protein, which vector also encodes a virus surface protein capable of binding specific target cell (e.g., cancer cells) surface proteins to direct the insertion of the IREN protein sequences into the cells. Further pharmaceutical compositions of the invention comprises as the active ingredient (a) an oligonucleotide sequence encoding an anti-sense sequence of the IREN protein sequence, or (b) drugs that block the IREN protein- TRAF interaction.

Pharmaceutical compositions according to the present invention include a sufficient amount of the active ingredient to achieve its intended purpose. In addition, the pharmaceutical compositions may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically and which can stabilize such preparations for administration to the subject in need thereof as are well known to those of skill in the art.

The IREN protein and its isoforms or isotypes are suspected to be expressed in different tissues at markedly different levels and apparently also with different patterns of isotypes in an analogous fashion to the expression of various other proteins involved in the intracellular signaling pathways as indicated in the above listed co-owned co-pending patent applications. These differences may possibly contribute to the tissue-specific features of response to the Fas/APO1-ligand and TNF. As in the case of other CED3/ICE homologs (Wang et al., 1994; Alnemri et al., 1995), the present inventors have previously shown (in the above mentioned patent applications) that MACH isoforms that contain incomplete

CED3/ICE regions (e.g., MA[illegible]α3) are found to have an inhibitory [illegible]t on the activity of co-expressed MACHα1 or MACHα2 molecules; they are also found to block death induction by Fas/APO1 and p55-R. Expression of such inhibitory isoforms in cells may constitute a mechanism of cellular self-protection against Fas/APO1- and TNF-mediated cytotoxicity. The wide heterogeneity of MACH isoforms, which greatly exceeds that observed for any of the other proteases of the CED3/ICE family, should allow a particularly fine-tuning of the function of the active MACH isoforms.

In accordance with the present invention there have also been isolated analogs/muteins of the TRAF2-binding protein IREN. Some of these IREN analogs/muteins (see above and see Examples below), such as deletion muteins of IREN modulate NF-κB activation. Hence, as noted above, the IREN proteins or possible isoforms may have varying effects in different tissues as regards their interaction with TRAF proteins and their influence thereby on the activity of the TRAF proteins, or intracellular signaling mediated by the TRAF proteins.

It is also possible that some of the possible IREN isoforms serve other functions. For example, IREN or some IREN analogs, or isoforms may also act as docking sites for molecules that are involved in other, non-cytotoxic effects of, for example, Fas/APO1 and TNF receptors via interaction with TRAF2 or even independently of TRAF2.

Due to the unique ability of Fas/APO1 and TNF receptors to cause cell death, as well as the ability of the TNF receptors to trigger other tissue-damaging activities, aberrations in the function of these receptors could be particularly deleterious to the organism. Indeed, both excessive and deficient functioning of these receptors have been shown to contribute to pathological manifestations of various diseases (Vassalli, 1992; Nagata and Golstein, 1995). Identifying the molecules that participate in the signaling activity of the receptors, and finding ways to modulate the activity of these molecules, could direct new therapeutic approaches. In view of the suspected important role of TRAF proteins, e.g. TRAF2 and hence the TRAF2-IREN interaction in modulation of NF-κB activation, it seems particularly important to design drugs that can modulate the TRAF2- IREN interaction when it is desired to kill cells (by inhibiting NF-κB activation), and conversely, when it is desired to preserve cells (by enhancement of NF-κB activation).

The present invention also concerns proteins or other ligands which can bind to the IREN proteins of the invention and thereby modulate/mediate the activity of the IREN proteins. Such proteins or ligands may be screened, isolated and produced by any of the above mentioned methods. For example, there may be isolated a number of new ligands, including

proteins, capable of binding to the IREN proteins of the invention (such new proteins/ligands excluding the known TRAF2 and TRAF1).

As detailed above, such new IREN protein-binding proteins/ligands, e.g. IREN-binding proteins, may serve as, for example, inhibitors or enhancers of IREN-mediated activity or the activity mediated by the, for example, TRAF2-IREN interaction, and as such will have important roles in various pathological and other situations as detailed above. Another function of such IREN protein-binding proteins/ligands would be to serve as specific agents for the purification of the IREN proteins by, for example, affinity chromatography, these new binding proteins/ligands being attached to the suitable chromatography matrices to form the solid or affinity support/matrix through which a solution, extract or the like, containing e.g. IREN, will be passed and in this way to facilitate the purification thereof. Such methods of affinity chromatography are now well known and generally standard procedures of the art.

Likewise, all of the above mentioned IREN proteins, analogs, fragments, isoforms and derivatives of the present invention may be used to purify by affinity chromatography the various TRAF proteins to which they bind. For example IREN, and analogs, fragments and muteins of IREN (see examples below) may be used for the affinity chromatography purification of TRAF2.

The invention will now be described in more detail in the following non-limiting examples and the accompanying drawings:

It should also be noted that the procedures of:
i) two-hybrid screen and two-hybrid β-galactosidase expression test; (ii) induced expression, metabolic labeling and immunoprecipitation of proteins; (iii) *in vitro* binding; (iv) assessment of the cytotoxicity; and (v) Northern and sequence analyses, as well as other procedures used in the following Examples have been detailed in previous publications by the present inventors in respect of other intracellular signaling proteins and pathways (see, for example, Boldin et al., 1995a, 1995b, and Boldin et al. 1996). These procedures also appear in detail in the co-owned co-pending Israel Application Nos. 114615, 114986, 115319, 116588, 117932, and 120367 as well as the corresponding PCT application No. PCT/US96/10521). Accordingly, the full disclosures of all these publications and patent applications are included herein in their entirety and at least as far as the detailed experimental procedures are concerned.

EXAMPLES

Materials and Methods

i) cDNA libraries

a) B-cell cDNA library

Oligo dT primed library constructed from human B cells was used (Durfee et al., 1993). The cDNAs of the library were inserted into the XhoI site of the pACT based vector pSE1107 in fusion with GAL4 activation domain.

b) λgt10 testis cDNA library

A cDNA library from human testis was used. The library is a random hexanucleotide primed library with an avarage insert size of 200 to 400 bp.

ii) Yeast strains

Two yeast stains were used as host strains for transformation and screening: HF7c strain that was used in the two hybrid screen and SFY526 strain that was used in the b-galactosidase assays. Both strains carry the auxotrophic markers trp1 and leu2, namely these yeast strains cannot grow in minimal synthetic medium lacking tryptophan and leucine, unless they are transformed by a plasmid carrying the wild-type versions of these genes (TRP1, LEU2). The two yeast strains carry deletion mutations in their GAL4 and GAL80 genes (gal4-542 and gal80-538 mutations, respectively).

SFY526 and HF7c stains carry the lacZ reporter in their genotypes; in SFY526 strain fused to the UAS and the TATA portion of GAL1 promoter, and in HF7c three copies of the GAL4 17-mer consensus sequence and the TATA portion of the CYC1 promoter are fused to lacZ. Both GAL1 UAS and the GAL4 17-mers are responsive to the GAL4 transcriptional activator. In addition, HF7c strain carries the HIS3 reporter fused to the UAS and the TATA portion of GAL1 promoter.

iii) Cloning of human TRAF2

The human TRAF2 was cloned by PCR from an HL60 cDNA library (for TRAF2 sequence and other details see Rothe et al., 1994; Rothe et al., 1995a; Cheng et al., 1996; Hsu et al., 1996; and Wallach, 1996). The primers used were: a) 30-mer forward primer CAGGATCCTCATGGCTGCAGCTAGCGTGAC corresponding to the coding sequence of hTRAF2 starting from the codon for the first Methionine (underlined) and including a linker with BamHI site. b) 32-mer reverse primer GGTCGACTTAGAGCCCTGTCAGGTCCACAATG that includes hTRAF2 gene stop

codon (underlined) a SalI restriction site in its linker. PCR program comprised of an initial denaturation step 2 min. at 94^0C followed by 30 cycles of 1 min. at 94^0C, 1 min. at 64^0C, 1 min. and 40 sec. at 72^0C. The amplified human TRAF2 was then inserted into the BamHI - SalI sites of pGBT9 vector in conjunction with GAL 4 DNA Binding domain.

iv) Two hybrid screen of B-cell library

The two hybrid screen is a technique (see details in above mentioned publications and patent applications) used in order to identify factors that are associated with a particular molecule that serves as a "bait". In the present invention TRAF2 that was cloned into the vector pGBT9, served as the bait. TRAF2 was co-expressed together with the screened B-cell cDNA library in the yeast strain HF7c. The PCR-cloned TRAF2 was a recombinant fusion with the CAL4 DNA-binding domain and the screened cDNA library was fused to the GAL4 activation domain in the pSE1107 vector. The reporter gene in HF7c was HIS3 fused to the upstream activating sequence (UAS) of the GAL1 promoter which is responsive to GAL4 transcriptional activator. Transformants that contained both pGBT9 and pSE1107 plasmids were selected for growth on plates without tryptophan and leucine. In a second step positive clones which expressed two hybrid proteins that interact with each other, and therefore activated GAL1-HIS3, were picked up from plates devoid of tryptophan, leucine and histidine and contained 50 mM 3-aminotriazol (3AT).

v) β-galactosidase assay

Positive clones picked up in the two hybrid screen were subjected to lacZ color development test in SFY526 yeast cells, following Clontech Laboratories' manual (for details see above mentioned publications and patent applications). In brief, transformants were allowed to grow at 30^0C for 2-4 days until reaching about 2 mm in diameter, then were transferred onto Whatman filters. The filters went through a freeze/thaw treatment in order to permeabilize the cells, then soaked in a buffer (16.1 mg/ml $Na_2HPO_4 \cdot 7H_2O$; 5.5 mg/ml $NaH_2PO_4 \cdot H_2O$; 0.75 mg/ml KCl; 0.75 mg/ml $MgSO_4 \cdot 7H_2O$, pH=7) containing 0.33 mg/ml X-gal and 0.35 mM β-mercaptoethanol. Colonies were monitored for development of blue color which is an indication for induction of β-galactosidase.

vi) Expression of cloned cDNAs

Two kinds of expression vectors were constructed:

a) A pUHD10-3 based vectors containing the open reading frame (ORF) of IREF in fusion with the Hemaglutinin (HA) epitope.

b) A pUHD10-3 based vec to which FLAG octapeptide sequen as introduced just in front of cloned TRAF2, hereby named FLAG/B6/TRAF2.

The constructs containing an ORF of IREN were transfected into HeLa-Bujard cells (for these cells see Gossen, M. and Bujard, M. (1992)) either alone or cotransfected with FLAG/B6/TRAF2 using standard calcium-phosphate method (Method in, for example, Current Protocols in Molecular Biology, eds. Ausubel, F.M et al.)

<u>vii) Luciferase assay</u>

Typically $5x10^5$ transfected cells were harvested by washing three times with cold PBS and resuspending in 400 µl extraction buffer (0.1 M K_2HPO_4/KH_2PO_4 pH=7.8; 1 mM DTT). Lysis of the cells was achieved by three times freezing in liquid nitrogen and thawing. Cell debris was removed by centrifugation (5 min. at 10,000 x g). For the luciferase assay, 200 µl of luciferase buffer (25 mM glycylglycine, 15 mM K_2HPO_4/KH_2PO_4 pH=7.8, 15 mM $MgSO_4$, 4 mM EGTA, 2 mM ATP, 1 mM DTT) were added to 50 µl of the lysate. Subsequently, 100 µl of 0.2 mM D-luciferine, 25 mM glycylglycine, 1 mM DTT were added to the reaction. Luciferase activity was determined by readind light emission using a Lumitron luminometer set on 10 seconds integration (see above publications and patent applications for additional details).

<u>Example 1: Cloning of IREN and two hybrid test</u>

A cDNA library prepared from B-cells was screened for proteins that associate with TRAF2, using the two-hybrid technique as described in Materials and Methods (iv). Only in transformants that expressed both TRAF2 and a protein capable of interacting with it, the GAL4 DNA-binding domain and the transcriptional activation domain were brought together. The result was the activation and expression of the reporter gene, in this case HIS3 fused to the UAS and the TATA portion of the GAL1 promoter.

The screen yielded approximately 2000 clones, which were able to grow on Trp-, Leu-, His- 3AT plates. DNA prepared from 165 randomly selected positive clones served for transient co-transfection of SFY526 yeast strain together with TRAF2 cloned into pGBT9 vector. Assay for β-galactosidase activity was performed on the transformed SFY526 yeast colonies as described in Materials and Methods (v). The blue color that developed was an indication for yeast colonies that contain cDNA encoding a protein or polypeptide that binds to TRAF2.

6 independent clones were identified that encoded the novel protein IREN by their ability to grow on 3AT plates and to induce LacZ as measured in the color test. Of all the positive clones checked, two were cDNAs coding for known proteins; TRAF2 itself that is capable of self-associating and forming homodimers, and the lymphotoxin beta receptor whose intracellular domains were shown to bind TRAF2.

The positive clones were further analysed in a binding specificity test, namely analysed for their interaction with irrelevant baits. As shown in Table II, IREN reacted only with TRAF2 and TRAF1 and did not bind to any one of a number of irrelevant proteins analysed such as lamin, and Cyclin D. IREN did not bind the intracellular domain of the p55 and p75 TNF receptors, MORT, NIK, NIK mutant $_{1\text{-}400}$, nor A20.

In order to narrow down the region on the TRAF2 molecule which interacts with IREN two additional constructs were made. One construct comprising the N-terminal part of the TRAF2 molecule, amino acids 1 to 224 designated RING $_{1\text{-}224}$ comprising the Ring finger and the zinc finger motifs. The second construct included only the C-terminal part of TRAF2, amino acids 225 to 501, covering the "TRAF-domain" as well as an additional 42 amino acids. These two constructs were used as baits in two hybrid tests. The results clearly show that IREN did not interact with the construct comprising amino acids 1 to 224 of the TRAF2 molecule, they did however bind to the C-terminal construct comprising the "TRAF domain" with the same efficiency as they bound to the full length TRAF2 (Table II). Deletion analysis demonstrated that the TRAF2 binding region in IREN and its isoforms is confined to the region between amino acids 198 and 388 thereof (Table II).

Table I[illegible]ast 2 hybrid test for IREN interac[illegible]s

Bait	Prey	Interaction
TRAF2	IREN	+++
TRAF2 $_{225\text{-}501}$	IREN	+++
RING $_{1\text{-}224}$	IREN	-
TRAF2	IREN$_{1\text{-}197}$	-
Lamin	IREN$_{1\text{-}197}$	-
TRAF2	IREN$_{198\text{-}388}$	+++
Lamin	IREN$_{198\text{-}388}$	-
TRAF2	IREN$_{398\text{-}541}$	+/-
Lamin	IREN$_{398\text{-}541}$	+/-
TRAF2	IREN$_{198\text{-}541}$	+++
Lamin	IREN$_{198\text{-}541}$	-
TRAF2	IREN 10B	++
IREN 10B	IREN 10B	++
IREN 10B	IREN	-
Lamin	IREN 10B	-
Lamin	IREN	-
CycD	IREN	-
p75IC	IREN	-
p55IC	IREN	-
MORT	IREN	-
TRAF3	IREN	-
NIK	IREN	-
NIK 1-400	IREN	-
TRAF1	IREN	+++
A20	IREN	-
TRAF6	IREN	-

In order to identify 5' sequences of IREN a phage pCEV cDNA library derived from the MCF7 cell line was used. The library is an oligodT-primed library that was screened with the first 600bp of IREN as probe. Preliminary sequencing of the insert of one of the clones indicated it contained a cDNA clone identical to IREN. The open reading frame of IREN cDNA encodes a protein of 541 amino acid. The cDNA also contains a short 3'UTR as well as poly(A)(Fig. 3A and 3B).

The 5' domain of IREN open reading frame (ORF) was found to contain a region which is homologous to one other known protein (ID: U73941, cloned in a 2-hybrid screen for Rap2 binding proteins (Janoueix-Lerosey I et al 1998) as well as to additional unknown proteins found in the databases: two human gene (KIAA0871, KIAA0842) and one C. Elegans gene (ID CAA21666).

The sequence of IREN was found to contain a peptide sequence [IDSLSL 326-331] which is also present within the 51 amino acid domain spanning amino acids 769 to 820 of NIK which is essential for IKK-1 binding to NIK in a 2-hybrid assay and NF-kB activation by NIK overexpression (data not shown).

Example 2: Further studies and functional characteristics of IREN

IREN cDNA fused to an HA epitope was expressed in the 293 human kidney cell lines using a pcDNA3 based vector containing the ORF of IREN in fusion with the Hemagglutinin (HA) epitope. IREN was then immunoprecipitated with anti HA antibodies. Cells were transfected with the IREN-HA fusion protein using a standard calcium-phosphate method (Method in, for example, Current Protocols in Molecular Biology, eds. Ausubel, F.M et al.). Cells were then grown for 24 hrs. in Dulbecco's Modified Eagle's Medium (DMEM) plus 10% calf serum. At the end of the incubation time, cells were lysed in radioimmune precipitation buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonident P-40, 1% deoxycholate, 0.1% SDS, and 1 mM EDTA; 1 ml/ $5x10^5$ cells), and the lysate was precleared by incubation with irrelevant rabbit antiserum and Protein G-Sepharose beads (Pharmacia, Sweden). Immunoprecipitation was performed by 1-hour incubation at 4°C of aliquots of the lysate with anti-HA (clone 12CA5 (Field, J. et al., 1988) monoclonal antibodies. The expressed proteins were analysed on an SDS-PAGE gel followed by Western Blot with anti HA antibodies. The protein encoded by IREN thus appears as a band of approximately 60kDa.

A multiple mRNA [illegible]e blot from Clontech Laboratories' [illegible]nalyzed according to manufacturer instructions. IREN is apparently expressed at low levels in tissues as the signal obtained in Northern blot analysis was found to be very low.

Studies of IREN effect on NF-κB activation were performed using the reporter gene assay. 293 EBNA cells were co-transfected with the pcDNA3 vector containing HIV LTR linked to the luciferase reporter gene, together with either the pcDNA3 plasmid containing IREN cDNA alone, or with a pcDNA3 plasmid containing the cDNA encoding the following proteins: IKK-1, full length NEMO, C-terminal deletion of NEMO, NIK, kinase deficient mutant of NIK (NIKmut), a C terminal deletion mutant of IREN ($IREN_{1-197}$) or an N-terminal deletion mutant of IREN ($IREN_{198-541}$).

pcDNA3 plasmids containing an ORF of IREN, were transfected into 293 cells either alone or cotransfected with the other pcDNA3 plasmids using a standard calcium-phosphate method (Method in, for example, Current Protocols in Molecular Biology, eds. Ausubel, F.M et al.) as described in the above Material and methods (vii).

In co-transfection with IKK-1, a known substrate for NIK enzymatic activity (Regnier CH, et al 1997), IREN was found to efficiently induce NF-κB in 293 cells as determined by the luciferase assay (see Fig. 10).

Co-transfection of a C-terminal deletion mutant of NEMO (CΔNEMO amino acids 1-309) which is reportedly able to block enzymatic activity of IKKs (Rothwarf DM, et al. 1998], was found to inhibit NF-κB induced by IREN and IKK1 co-transfection (Fig. 10).

The activity of NF-κB induced by IREN and IKK1 co-transfection was comparable to that induced by co-expression of full size NEMO and IKK1. NEMO was not able to further potentiate NF-κB induced by IREN and IKK1 (Fig. 10).

Unlike CΔNEMO, cotransfection of a kinase-deficient mutant of NIK (NIKmut, amino acids (see co-pending co-owned Patent Application WO 97/37016, Malinin et al. 1996) was not able to block NF-kB induction by cotransfection of IREN and IKK1 (Fig. 10).

IREN co-expressed at a 2:1 fold ratio with NIK DNA effectively potentiates induction of NF-κB. Co-expression of full-size NEMO with NIK blocks NF-κB induced by the latter. This inhibition could be reversed by expression of IREN, however the inhibition imposed by CΔNEMO was unaffected by co-expression of IREN (Table I).

As it was shown earlier (see co-pending co-owned Patent Application WO 97/37016, Malinin et al. 1996) NIKmut effectively blocks NF-κB induced by CD120a overexpression. This effect was not altered by co-expression of IREN (Fig. 10).

Taken together the abovementioned data lead to the suggestion that IREN acts within the NF-κB signalling pathway, acting downstream to NIK but upstream to NEMO and IKK1 and could be a modulator of the interaction between NIK and IKK-1.

It was therefore hypothesized that some deletion mutant of IREN might interfere with the signal flow from NIK kinase to the NEMO-IKK complex. The C terminal deletion mutant $IREN_{1-197}$ did not have any effect on NIK-induced NF-kB activation (data not shown). However an N-terminal deletion mutant of IREN ($IREN_{198-541}$) profoundly inhibited NIK-induced NF-κB, to the extent comparable to that of NEMO (Fig. 10).

Example 3: Kinase Assay

293-T cells ($2x10^6$) were transfected with pcFLAG CHUK (encoding murine IKK1) alone or in combination with pcNIK, pc20.4 (encoding the NEMO protein) or pcHIS-IREN (encoding His tagged-IREN), or pcHIS-IRENΔN (pcHIS-IREN $_{198-541}$ encoding a His tagged N terminal deletion mutant of IREN that acts as a dominant negative). 24 h post transfection cells were harvested, lysed in lysis buffer containing 1% NP-40, 50 mM Hepes pH-7.5, 100 mM NaCl, 10% glycerol, 1mM EDTA, 20 mM b-glycerophosphate, 20 mM PNPP, 1 mM Na3VaO4, 1 mM NaF, 1 mM Na-Metabisulfite, 1 mM Bezamidine, 1 mM DTT, "Complete" protease inhibitors (Boehringer).
Cell debris was then removed by centrifugation. Following addition of NaCl to up to 250 mM, proteins were immunoprecipitated with monoclonal anti FLAG antibodies, washed thoroughly in washing buffer containing lysis buffer with 0.1% NP-40 and 250 mM NaCl, and eluted with 30 μl wash buffer containing 1 mg/ml FLAG peptide. Aliquots of the eluates were used for an *in-vitro* kinase reactions with E. coli produced GST-IκB as substrate, in the presence of ^{32}P-gamma ATP in kinase buffer containing 50 mM β–glycerophosphate, 2 mM DTT, 20 mM $MgCl_2$, 1 mM Na_3 VaO_4, 1 mM EDTA /EGTA. The reactions were separated by SDS-PAGE and phosphorylation of proteins was detected after exposure to X-Ray film. As control, amount of protein in the lysate was determined by western blot with anti FLAG antibody.

The N terminal deletion mutant of IREN was found to act as a dominant negative molecule and to block IKK-1 activity in the kinase assay when IREN and IKK-1 were coexpressed with NEMO.

Overexpression of IKK1 and NEMO, but not of IKK1 alone, induces robust kinase activity of IKK1 (as assessed by autophosphorylation and by phosphorylation of E.coli produced GST-IkappaB fusion protein). Coexpression of IREN $_{198\text{-}541}$ with IKK1 and NEMO results in a significant decrease in activity (Fig. 11) without affecting the IKK1 and NEMO expression level. Full size IREN did not have such an effect (Fig. 11, middle lane).

Example 4: Cloning and sequencing of IREN-10B and IREN-E

In order to identify splice variants of IREN, a phage cDNA library derived from the abovementioned MCF7 cell line was screened with the first 600bp of IREN as probe. Two independent clones were identified which appeared to be two different variants of IREN. These two clones are identical to IREN in their first 5' 1595 bp and have additional coding sequences at the 3-prime end. This region contains a PX domain – a conservative domain of unknown function (presumably a protein-protein interaction domain) that is also found in some signalling molecules, including PI3-kinase. In clone 10B and clone E the PX domain is flanked by two short identical regions. The region downstream to these regions is different in the two clones. For a comparison of the two splice variants to IREN see Fig. 9.

Provisional sequencing of the 5-prime UTR (from the beginning of the sequence up to the first ATG with Kozak sequence) indicated these sequences were identical in IREN and in IREN-10B and IREN-E isoforms.

The region of TRAF2 binding is mapped to IREN $_{198\text{-}388}$, which is identical in all three splice isoforms shown (Fig. 9). Accordingly, IREN 10B also interacted with TRAF2 in the two-hybrid assay. Although no self-association of IREN was observed, IREN 10B did self-associate in the two-hybrid test, whereas, it did not interact with IREN (Table II). These data confirm that the PX domain is probably the region of protein self-interaction.

REFERENCES

1. Adelman et al., (1983) DNA 2, 183.
2. Alnemri, E.S. et al. (1995) J. Biol. Chem. 270, 4312-4317.

3. Arch RH et al (1998). Genes Dev. Sep 15;12(18):2821-30

4. Arch, R.H. and C.B. Thompson. (1998) B. *Mol. Cell. Biol.* 18, 558-565.

5. Ausubel. F.M. et al. eds.. Current Protocols in Molecular Biology.

6. Baeuerle, P. A., and Henkel, T. (1994) Annu Rev Immunol.

7. Bazan, J. F. (1993). Current Biology 3, 603-606.

8. Berberich, I., Shu, G. L., and Clark, E. A. (1994). J Immunol 153, 4357-66.

9. Beutler, B., and van Huffel, C. (1994). Science 264, 667-8.

10. Blank, V., Kourilsky, P.. and Israel, A. (1992). Trends Biochem. Sci 17, 135-40.

11. Boldin, M.P. et al. (1995a) J. Biol. Chem. 270, 337-341.

12. Boldin, M. P., Varfolomeev, E. E., Pancer, Z., Mett, I. L., Camonis, J. H., and Wallach, D. (1995b). J. Biol. Chem. 270, 7795-7798.

13. Boldin, M.P. et al. (1996) Cell 85, 803-815.

14. Born TL et al. (1998) J Biol Chem. 273, 29445-50.

15. Cao, Z. et al. (1996a) Nature 383, 443-446.

16. Cao, Z. et al. (1996b) Science 271, 1128-1131.

17. Chen, C.J. et al. (1992) Ann. N.Y. Acad. Sci. 660:271-273.

18. Cheng, G., Cleary, A.M., Ye, Z-s., Hong, D.I., Lederman, S. and Baltimore, D. (1995) Science 267:1494-1498.

19. Cheng, G. and Baltimore, D. (1996) Genes Dev. 10, 963-973.

20. Chinnalyan, A. M., O'Rourke, K., Tewari, M., and Dixit, V. M. (1995) Cell 81, 505-512.

21. Creighton, T.E., Proteins : Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, Ca. 1983.

22. Croston, G. E., Cao, Z., and Goeddel, D. V. (1995). J Biol Chem 270, 16514-7.

23. DiDonato, J. A., Mercurio, F., and Karin, M. (1995). Mol Cell Biol 15, 1302-11.

24. Duckett, C.S., R.W. Gedrich, M.C. Gilfillan, and C.B. Thompson. (1997) *Mol. Cell. Biol.* 17, 1535-1542

25. Durfee, T. et al. (1993) Genes Dev. 7:555-569.

26. Field, J. et al. (1988) Mol. Cell Biol. 8:2159-2165.

27. Geysen, H.M. (1985) Immunol. Today 6, 364-369.

28. Geysen, H.M. et al. (1987) J. Immunol. Meth. 102, 259-274.

29. Gilmore, T. D., and Morin, P. J. (1993). Trends Genet 9, 427-33.

30. Gossen, M. and Bujard, M. (1992) PNAS 89:5547-5551.

31.Grell, M., Douni, E., Wajant, H., Lohden, M., Clauss, M., Baxe[illegible] B., Georgopoulos, S., Lesslauer, W., Kollias, G., Pfizenmaier, K., and Scheurich, P. (1995). Cell 83, 793-802.

32.Grilli, M., Chiu, J. J., and Lenardo, M. J. (1993). Int RevCytol.

33.Hanks, S. K., Quinn, A. M., and Hunter, T. (1988). Science 241, 42-52.

34.Hibi, M., Lin, A. Smeal,T. Minden, A. and Karin M.(1993) *Genes & Dev.*7, 2135-2148

35.Howard, A.D. et al. (1991) J. Immunol. 147, 2964-2969.

36.Hsu, H., Shu, H.-B., Pan, M.-G., and Goeddel, D. V. (1996). Cell 84, 299-308.

37.Hsu, H., Xiong, J., and Goeddel, D. V. (1995). Cell 81, 495-504.

38.Ishida T et al. (1996a) Proc Natl Acad Sci U S A. 93, 9437-42.

39.Ishida T et al. (1996b) J Biol Chem. 271, 28745-8.

40.Janoueix-Lerosey I et al Eur J Biochem (1998) Mar 1;252(2):290-8

41.Kanakaraj P et al (1999) J Exp Med 189, 1129-38

42.Kaufmann, S.H. (1989) Cancer Res. 49, 5870-5878.

43.Kaufmann, S.H. (1993) Cancer Res. 53, 3976-3985.

44.Lalmanach-Girard, A. C., Chiles, T. C., Parker, D. C., and Rothstein, T. L. (1993). J Exp Med 177, 1215-1219.

45.Lazebnik, Y.A. et al. (1994) Nature 371, 346-347

46.Lee, S.Y., C.G. Park, and Y. Choi. 1996 J. Exp. Med. 183, 669-674.

47.Lee, S.Y., A. Reichlin, A. Santana, K.A. Sokol, M.C. Nussenzweig, and Y. Choi. (1997) Immunity 7, 703-713.

48.Li Y et al (1998) Mol Cell Biol. 18, 1601-10.

49.Lin, A., A. Minden, H. Martinetto, F.X. Claret, C. Lange-Carter, F. Mercurio, G.L. Johnson, and M. Karin. (1995) *Science* 268, 286-290.

50.Liu, Z.-G., Hsu, H. Goeddel D.V., and Karin M.. 1996. *Cell* 87, 565-576

51.Mashima, T. et al. (1995) Biochem. Biophys. Res. Commun. 209, 907-915.

52.McDonald, P. P., Cassatella, M. A., Bald, A., Maggi, E., Romagnani, S., Gruss, H. J., and Pizzolo, G. (1995). Eur J Immunol 25, 2870-6.

53.Mercurio F and Manning AM (1999a) Curr Opin Cell Biol. 1999 11,226-32

54.Mercurio F et al (1999b) Mol Cell Biol. 19, 1526-38.

55.Messing et al., Third Cleveland Symposium on Macromolecules and Recombinant DNA, Ed. A. Walton, Elsevier, Amsterdam (1981)

56.Minden, A., Lin A., McMahon M., Lange-Carter C., Derijard B., Davis R.J., Johnson, G.L. and. Karin M. 1994. *Science* 266, 1719-1723.

57.Milligan. C.E. et al. (1995) Neuron 15, 385-393.

58.Mosialos, G., Birkenbach, M., Yalamanchili, R., VanArsdale, T., Ware, C., and Kieff, E. (1995). Cell 80, 389-399.

59.Muranishi, S. et al. (1991) Pharm. Research 8, 649.

60.Nagata, S. and Golstein, P. (1995) Science 267, 1449-1456.

61.Nakano H (1996) J Biol Chem. 271, 14661-4.

62.Park, Y.C. (1999) Nature. 398, 533-8

63.Reinhard C., Shamoon, B.Shyamala,V. and Williams.L.T.1997. *EMBO J.* 16, 1080-1092

64.Régnier CH (1995) J Biol Chem. 270, 25715-21.

65.Regnier CH, Song HY, Gao X, Goeddel DV, Cao Z, Rothe M (1997) Cell 90, 373-383

66.Rensing-Ehl, A., Hess, S., Ziegler-Heitbrock, H. W. L., Riethmüller, G., and Engelmann, H. (1995). J. Inlamm. 45, 161-174.

67.Rothe, M., Pan, M-G, Henzel, W. J., Ayres, T. M., and Goeddel, D. V. (1995b). Cell 83, 1243-1252.

68.Rothe, M., Sarma, V., Dixit, V. M., and Goeddel, D. V. (1995a). Science 269, 1424-1427.

69.Rothe, M., Wong, S. C., Henzel, W. J., and Goeddel, D. V. (1994). Cell 78, 681-692.

70.Rothe, M. et al. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 8241-8246.

71.Rothwarf DM et al (1998) Nature. 395,297-300.

72.Roy, N., Q.L. Deveraux, R. Takahashi, G.S. Salvesen, and J.C. Reed. (1997). EMBO J. 16, 6914-6925.

73.Ruzicka et al., (1993) Science 260, 487.

74.Sambrook et al. (1989) Molecular cloning: a laboratory manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

75.Sano et al., (1992) Science 258, 120.

76.Sano et al., (1991) Biotechniques 9, 1378.

77.Schreiber, E., Matthias, P., Muller, M.M. and Schaffner, W. (1989), Nuc. Acids Res. 17:6419.

78.Schulz et al., G.E., Principles of Protein Structure, Springer-Verlag, New York, N.Y. 1798.

79.Sleath, P.R. et al. (1990) J. Biol. Chem. 265,14526-14528.

80.Shi, C.-S. and J.H. Kehrl. (1997) *J. Biol. Chem.* 272, 32102-32107.

81.Smith. C. A.. Farrah. T., and Goodwin, R. G. (1994). Cell 76, 959

82.Song, H.Y., C.H. Régnier, C.J. Kirschning, D.V. Goeddel, and M. Rothe. (1997). *Proc. Natl. Acad. Sci.* 9, 9792-9796

83.Stanger. B.Z. et al. (1995) Cell 81, 513-523.

84.Su. B.. E. Jacinto. M. Hibi. T. Kallunki. M. Karin, and Y. Ben-Neriah. (1994) *Cell* 77. 727-736

85.Thornberry, N.A. et al. (1992) Nature 356,768-774.

86.Thornberry, N.A. et al. (1994) Biochemistry 33, 3934-3940.

87.Vandenabeele, P., Declercq, W., Beyaert, R., and Fiers, W. (1995). Trends Cell Biol. 5, 392-400.

88.Varfolomeev, E. E., Boldin, M. P., Goncharov, T. M., and Wallach, D.

89.(1996). J. Exp. Med. 183, 1271-5.

90.Vassalli, P. (1992) Ann. Rev. Immunol. 10, 411-452.

91.Veira et al., (1987) Meth. Enzymol. 153, 3.

92. Wallach, D. (1996) Eur. Cytokine Net. 7, 713-724.

93. Wang, L. et al. (1994) Cell 78, 739-750.

94. Wilks, A.F. et al. (1989) Proc. Natl. Acad. Sci. USA, 86:1603-1607.

95.Yamaoka S et al. (1998) Cell. 93, 1231-40.

96.Yeh, W.-C., A. Shahinian, D. Speiser, J. Kraunus, F. Billia, A. Wakeham, J.L. de la Pompa, D. Ferrick, B. Hum, N. Iscove, P. Ohashi, M. Rothe, D.V. Goeddel, and T.W. Mak. (1997) *Immunity* 7, 715-725

97. Zaccharia, S. et al. (1991) Eur. J. Pharmacol. 203, 353-357.

98. Zhao, J.J. and Pick, L. (1993) Nature 365: 448-451.

CLAIMS

1. A DNA sequence encoding a protein capable of binding to TRAF selected from the group consisting of:

(a) a cDNA sequence of the herein designated IREN comprising the nucleotide sequence depicted in Fig. 3;

(b) a cDNA sequence of the herein designated IREN-10B comprising the nucleotide sequence depicted in Fig. 4;

(c) a cDNA sequence of the herein designated IREN-E comprising the nucleotide sequence depicted in Fig. 5;

(d) a fragment of a sequence (a)-(c) which encodes a biologically active protein capable of binding to at least the 225-501 amino acid sequence of TRAF2;

(e) a DNA sequence capable of hybridization to a sequence of (a)-(d) under moderately stringent conditions and which encodes a biologically active protein capable of binding to at least the 225-501 amino acid sequence of TRAF2; and

(f) a DNA sequence which is degenerate as a result of the genetic code to the DNA sequences defined in (a)-(e) and which encodes a biologically active protein capable of binding to at least the 225-501 amino acid sequence of TRAF2.

2. A DNA sequence according to claim 1, selected from the cDNA sequences herein designated IREN and IREN-10B and IREN-E.

3. A DNA sequence according to any one of claims 1 or 2, which DNA encodes a protein that modulates NF-κB activity.

4. A DNA sequence according to claim 6, selected from the sequences contained in the herein-designated cDNA IREN.

8. A DNA sequence according to claim 1 or 6, comprising the DNA sequence encoding the protein IREN (as herein defined).

9. A DNA sequence encoding the protein IREN, isoforms, fragments or analogs thereof, said IREN, isoforms, fragments or analogs thereof being capable of binding to TRAF2 and which is capable of modulating the activity of NF-κB.

10. A DNA sequence according to claim 9, selected from the group consisting of:

a) a cDNA sequence derived from the coding region of a native IREN protein;

b) DNA sequences capable of hybridization to a sequence of (a) under moderately stringent conditions and which encode a biologically active IREN; and

c) DNA sequences which are degenerate as a result of the genetic code to the sequences defined in (a) and (b) and which encode a biologically active IREN protein.

11. A DNA sequence according to claim 9 or 10 comprising at least part of the sequence depicted in Fig. 6 and encoding at least one active IREN protein, isoform, analog or fragment.

12. A DNA sequence according to claim 11 encoding a IREN protein, isoform, analog or fragment having at least part of the amino acid sequence depicted in Fig. 6.

13. A vector comprising a DNA sequence according to any one of claims 1-12.

14. A vector according to claim 13 capable of being expressed in a eukaryotic host cell.

15. A vector according to claim 13 capable of being expressed in a prokaryotic host cell.

16. Transformed eukaryotic or prokaryotic host cells containing a vector according to any one of claims 13-15.

17. An IREN protein, isoforms, fragments, analogs and derivatives thereof, encoded by a DNA sequence according to any one of claims 1-12, said protein, isoforms, fragments, analogs and derivatives thereof being capable of binding to at least the portion of the TRAF2 protein between amino acids 225-501 of TRAF2.

18. A protein according to claim 17 being the protein encoded by herein-designated clone 10B.

19. A protein, isoforms, fragments, analogs and derivatives thereof according to claim 17 being the protein IREN, isoforms, analogs, fragments and derivatives thereof encoded by the DNA sequence according to any one of claims 1-12.

20. A protein IREN, isoforms, analogs, fragments and derivatives thereof according to claim 19, wherein said protein, isoforms, fragments and derivatives have at least part of the amino acid sequence depicted in Fig. 6.

21. A method for producing a protein, isoform, fragment, analog or derivative thereof according to any one of claims 17-19, which comprises growing a transformed host cell according to claim 16 under conditions suitable for the expression of said protein, isoform, fragment, analog or derivative thereof, effecting post-translational modification, as necessary, for obtaining said protein, isoform, fragment, analog or derivative thereof, isolating said expressed protein, isoform, fragment, analog or derivative.

22. Antibodies or active fragments or derivatives thereof, specific for the IREN protein. isoform, analog, fragment or derivative thereof according to claim 17 or 18; or specific for the protein IREN. isoform, analog, fragment or derivative thereof according to claim 19 or 20.

23. A method for the modulation or mediation in cells of the activity of NF-κB or any other intracellular signaling activity modulated or mediated by TRAF2 or by other molecules to which a protein, isoform, analog, fragment or derivative thereof according to any one of claims 17-20 binds, said method comprising treating said cells by introducing into said cells one or more of said protein, isoform, analog, fragment or derivative thereof in a form suitable for intracellular introduction thereof, or introducing into said cells a DNA sequence encoding said one or more protein, isoform, analog, fragment or derivative thereof in the form of a suitable vector carrying said sequence, said vector being capable of effecting the insertion of said sequence into said cells in a way that said sequence is expressed in said cells.

24. A method according to claim 23, wherein said treating of cells comprises introducing into said cells a DNA sequence encoding said protein, isoform, fragment, analog or derivative in the form of a suitable vector carrying said sequence, said vector being capable of effecting the insertion of said sequence into said cells in a way that said sequence is expressed in said cells.

25. A method according to claim 23 or 24 wherein said treating of said cells is by transfection of said cells with a recombinant animal virus vector comprising the steps of:

(a) constructing a recombinant animal virus vector carrying a sequence encoding a viral surface protein (ligand) that is capable of binding to a specific cell surface receptor on the surface of said cells to be treated and a second sequence encoding a protein selected from the said protein, isoforms, analogs, fragments and derivatives according to any one of claims 17-20, that when expressed in said cells is capable of modulating/mediating the activity of NF-κB or any other intracellular signaling activity modulated/mediated by TRAF2 or other said molecules; and

(b) infecting said cells with said vector of (a).

26. A method for modulating TRAF2 modulated/mediated effect on cells comprising treating said cells with antibodies or active fragments or derivatives thereof, according to claim 22, said treating being by application of a suitable composition containing said antibodies, active fragments or derivatives thereof to said cells, wherein when the IREN protein or portions thereof of said cells are exposed on the extracellular surface, said

composition is formulated [illegible]xtracellular application, and when IREN proteins are intracellular said composition is formulated for intracellular application.

27. A method for modulating the TRAF2 modulated/mediated effect on cells comprising treating said cells with an oligonucleotide sequence encoding an antisense sequence for at least part of the DNA sequence encoding an IREN protein according to any one of claims 1-11, said oligonucleotide sequence being capable of blocking the expression of the IREN protein.

28. A method according to claim 27 wherein said oligonucleotide sequence is introduced to said cells via a virus of claim 25 wherein said second sequence of said virus encodes said oligonucleotide sequence.

29. A method for modulating the TRAF2 modulated/mediated effect on cells comprising applying the ribozyme procedure in which a vector encoding a ribozyme sequence capable of interacting with a cellular mRNA sequence encoding an IREN protein according to any one of claims 17-20, is introduced into said cells in a form that permits expression of said ribozyme sequence in said cells, and wherein when said ribozyme sequence is expressed in said cells it interacts with said cellular mRNA sequence and cleaves said mRNA sequence resulting in the inhibition of expression of said IREN protein in said cells.

30. A method for isolating and identifying proteins, according to any one of claims 17-20, capable of binding directly to TRAF2, comprising applying the yeast two-hybrid procedure in which a sequence encoding said TRAF2 is carried by one hybrid vector and sequence from a cDNA or genomic DNA library is carried by the second hybrid vector, the vectors then being used to transform yeast host cells and the positive transformed cells being isolated, followed by extraction of the said second hybrid vector to obtain a sequence encoding a protein which binds to said TRAF2.

31. A method according to any one of claims 23-30 wherein said protein is IREN or at least one of the IREN isoforms, analogs, fragments and derivatives thereof.

32. A pharmaceutical composition for the modulation of the TRAF2 modulated/mediated effect on cells comprising, as active ingredient at least one IREN protein, according to any one of claims 17-20, its biologically active fragments, analogs, derivatives or mixtures thereof.

33. A pharmaceutical composition for modulating the TRAF2 modulated/mediated effect on cells comprising, as active ingredient, a recombinant animal virus vector encoding a

protein capable of binding a cell surface receptor and encoding at least one IREN protein, isoform, active fragments or analogs, according to any one of claims 17-20.

34. A pharmaceutical composition for modulating the TRAF2 modulated/mediated effect on cells comprising as active ingredient, an oligonucleotide sequence encoding an anti-sense sequence of the IREN protein mRNA sequence according to any one of claims 1-11.

35. A pharmaceutical composition for the prevention or treatment of a pathological condition associated with NF-κB induction or with any other activity mediated by TRAF2 or by other molecules to which a protein according to any one of claims 17-20 binds, said composition comprising an effective amount of a protein encoded by IREN-10B or a DNA molecule coding therefor, or a molecule capable of disrupting the interaction of said protein encoded by IREN-10B with TRAF2 or any other molecule to which a protein encoded by IREN-10B binds.

36. A pharmaceutical composition for the prevention or treatment of a pathological condition associated with NF-κB induction or with any other activity mediated by TRAF2 or by other molecules to which a protein according to any one of claims 17-20 binds, said composition comprising an effective amount of a protein IREN, isoform, fragment, analog or derivative thereof, or a DNA molecule coding therefor, or a molecule capable of disrupting the interaction of said protein IREN, isoform, fragment, analog or derivative thereof with TRAF2 or any other molecule to which said protein IREN, isoform, fragment, analog or derivative binds.

37. A pharmaceutical composition for the prevention or treatment of a pathological condition associated with NF-κB induction or with any other activity mediated by TRAF2 or by other molecules to which the protein IREN binds, said composition comprising a molecule capable of interfering with the activity of the protein IREN.

38. A pharmaceutical composition for the prevention or treatment of a pathological condition associated with NF-κB induction or with any other activity mediated by TRAF2 or by other molecules to which a protein encoded by IREN-10B according to claim 18 binds, said composition comprising an effective amount of a protein encoded by IREN 10B or a DNA molecule coding therefor, or a molecule capable of disrupting the interaction of said protein encoded by IREN 10B with TRAF2 or any other molecule to which said protein encoded by IREN 10B binds.

39. A pharmaceutical composition for the prevention or treatment of a pathological condition associated with NF-κB induction or with any other activity mediated by TRAF2 or by other molecules to which a protein IREN, isoform, fragment, analog or derivative according to claim 19 or 20 binds, said composition comprising an effective amount of a protein IREN, isoform, fragment, analog or derivative thereof, or a DNA molecule coding therefor, or a molecule capable of disrupting the interaction of said protein IREN, isoform, fragment, analog or derivative thereof with TRAF2 or any other molecule to which said protein IREN, isoform, fragment, analog or derivative binds.

40. A method for the prevention or treatment of a pathological condition associated with NF-κB induction or with any other activity mediated by TRAF2 or by other molecules to which a protein according to any one of claims 17-20 binds, said method comprising administering to a patient in need an effective amount of a protein or isoform, fragment, analog and derivative thereof or a mixture of any thereof according to any one of claims 17-20, or a DNA molecule coding therefor, or a molecule capable of disrupting the interaction of said protein or isoform, fragment, analog and derivative thereof or a mixture of any thereof according to any one of claims 17-20 with TRAF2 or any other molecule to which said protein or isoform, fragment, analog and derivative thereof or a mixture of any thereof according to any one of claims 17-20 binds.

41. A method according to claim 40 wherein said protein is encoded by IREN.

42. A method according to claim 40, wherein said protein is IREN.

43. A method for screening of a ligand capable of binding to a protein according to any one of claims 17-20 comprising contacting an affinity chromatography matrix to which said protein is attached with a cell extract whereby the ligand is bound to said matrix, and eluting, isolating and analyzing said ligand.

44. A method for screening of a DNA sequence coding for a ligand capable of binding to a protein according to any one of claims 17-20 comprising applying the yeast two-hybrid procedure in which a sequence encoding said protein is carried by one hybrid vector and sequences from a cDNA or genomic DNA library are carried by the second hybrid vector, transforming yeast host cells with said vectors, isolating the positively transformed cells, and extracting said second hybrid vector to obtain a sequence encoding said ligand.

45. A method for identifying and producing a ligand capable of modulating the cellular activity modulated/mediated by TRAF2 comprising:

a) Screening for a ligand capable of binding to a polypeptide comprising at least a portion of TRAF2 having the amino acid residues 225-501 of TRAF2;

b) Identifying and characterizing a ligand, other than TRAF2 or portions of a receptor of the TNF/NGF receptor family, found by said screening step to be capable of said binding; and

c) Producing said ligand in substantially isolated and purified form.

46. A method for identifying and producing a ligand capable of modulating the cellular activity modulated or mediated by a protein according to any one of claims 17-20 comprising :

a) Screening for a ligand capable of binding to a polypeptide comprising at least a portion of the sequence IREN depicted in Fig. 6;

b) Identifying and characterizing a ligand, other than TRAF2 or portions of a receptor of the TNF/NGF receptor family, found by said screening step to be capable of said binding; and

c) Producing said ligand in substantially isolated and purified form.

47. A method for identifying and producing a ligand capable of modulating the cellular activity modulated/mediated by the protein IREN comprising:

a) Screening for a ligand capable of binding to at least a portion of the IREN sequence depicted in Fig. 6;

b) Identifying and characterizing a ligand, other than TRAF2 or portions of a receptor of the TNF/NGF receptor family, found by said screening step to be capable of said binding; and

c) Producing said ligand in substantially isolated and purified form.

48. A method for identifying and producing a molecule capable of directly or indirectly modulating the cellular activity modulated/mediated by the protein IREN, comprising:

a) Screening for a molecule capable of modulating activities modulated/mediated by the protein IREN;

b) Identifying and characterizing said molecule; and

c) Producing said molecule in substantially isolated and purified form.

49. A method for identifying and producing a molecule capable of directly or indirectly modulating the cellular activity modulated/mediated by a protein according to any one of claims 17-20, comprising:

a) Screening for a molecule capable of modulating activities modulated/mediated by a protein according to any one of claims 17-20;

b) Identifying and characterizing said molecule; and

c) Producing said molecule in substantially isolated and purified form.

For the Applicant
Henry Einav

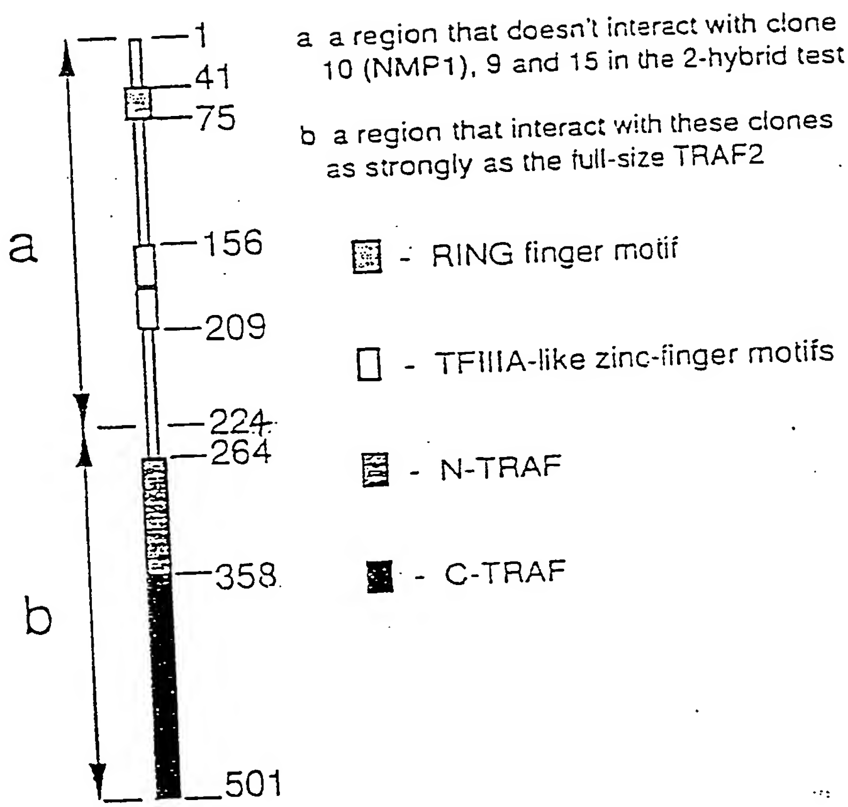

1
41
75
156
209
224
264
358
501
a
b
a a region that doesn't interact with clone 10 (NMP1), 9 and 15 in the 2-hybrid test
b a region that interact with these clones as strongly as the full-size TRAF2
- RING finger motif
- TFIIIA-like zinc-finger motifs
- N-TRAF
- C-TRAF

Figure 1

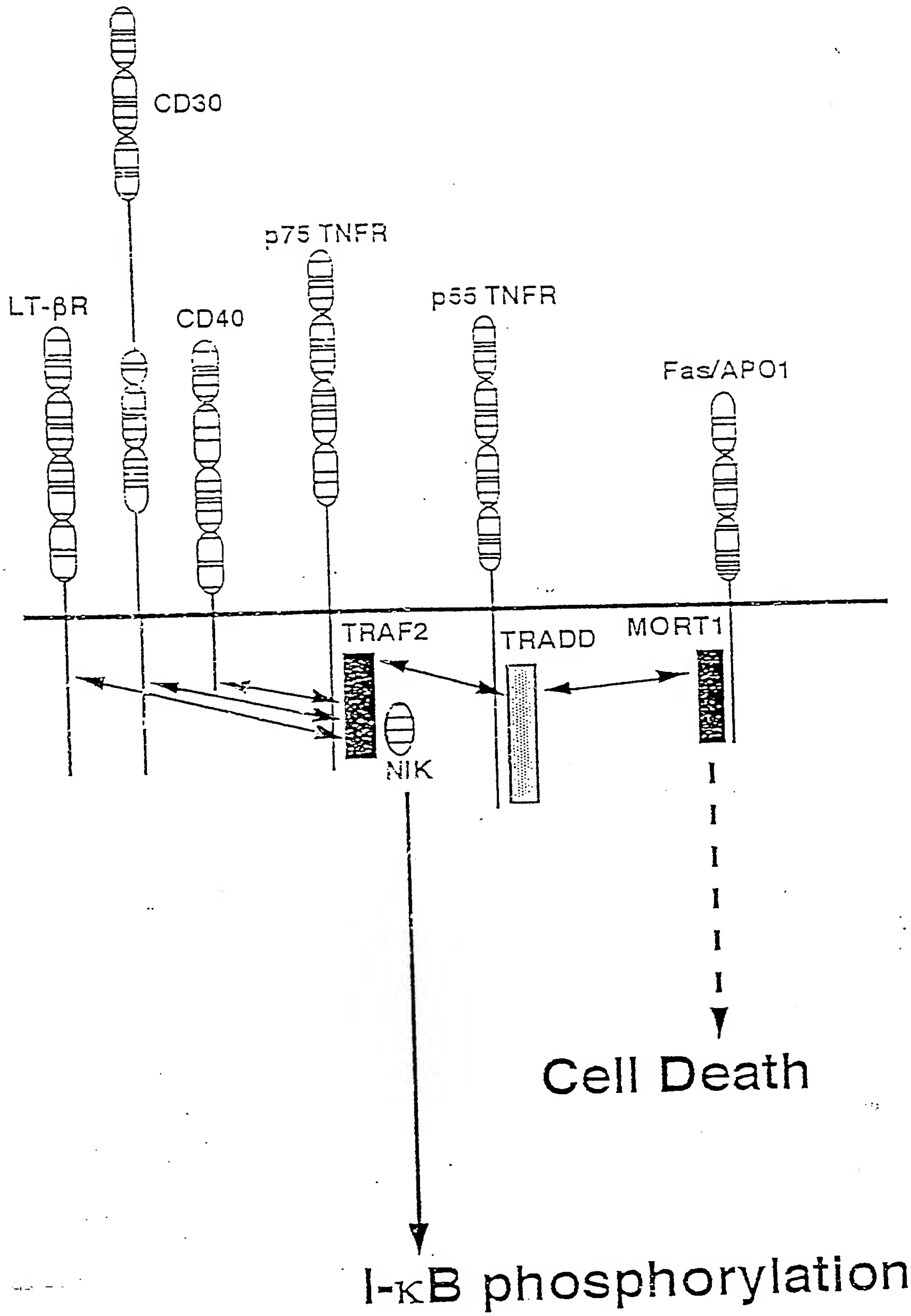

CD30
p75 TNFR
LT-βR
CD40
p55 TNFR
Fas/APO1
TRAF2
TRADD
MORT1
NIK
Cell Death
I-κB phosphorylation

Figure 2

GGTACCGAGCTCGGATCCACTAGTAACGGCCGCCAGTGTGCTGGAATTCTGCGGATGTACCCATACGATGTTCCAGATA
CGCTGAATTTCGAGGCCACGAAGGCCGGCGGCGCGGCGCAGGCACCGGCCCGGGGAGAGGCACC

Figure 3A: Nucleotide sequence of IRENs 5'UTR

ATG AGC GGA TCA CAG AAC AAT GAC AAA AGA CAA TTT CTG CTG GAG CGA [illegible]G CTG GAT GCA
61 91
GTG AAA CAG TGC CAG ATC CGC TTT GGA GGG AGA AAG GAG ATT GCC TCG GAT TCC GAC AGC
121 151
AGG GTC ACC TGT CTG TGT GCC CAG TTT GAA GCC GTC CTG CAG CAT GGC TTG AAG AGG AGT
181 211
CGA GGA TTG GCA CTC ACA GCG GCA GCG ATC AAG CAG GCA GCG GGC TTT GCC AGC AAA ACC
241 271
GAA ACA GAG CCC GTG TTC TGG TAC TAC GTG AAG GAG GTC CTC AAC AAG CAC GAG CTG CAG
301 331
CGC TTC TAC TCC CTG CGC CAC ATC GCC TCA GAC GTG GGC CGG GGT CGC GCC TGG CTG CGC
361 391
TGT GCC CTC AAC GAA CAC TCC CTG GAG CGC TAC CTG CAC ATG CTC CTG GCC GAC CGC TGC
421 451
AGG CTG AGC ACT TTT TAT GAA GAC TGG TCT TTT GTG ATG GAT GAA GAA AGG TCC AGT ATG
481 511
CTT CCT ACC ATG GCA GCA GGT CTG AAC TCC ATA CTC TTT GCG ATT AAC ATC GAC AAC AAG
541 571
GAT TTG AAC GGG CAG AGT AAG TTT GCT CCC ACC GTT TCA GAC CTC TTA AAG GAG TCA ACG
601 631
CAG AAC GTG ACC TCC TTG CTG AAG GAG TCC ACG CAA GGA GTG AGC AGC CTG TTC AGG GAG
661 691
ATC ACA GCC TCC TCT GCC GTC TCC ATC CTC ATC AAA CCT GAA CAG GAG ACC GAC CCC TTG
721 751
CCT GTC GTG TCC AGG AAT GTC AGT GCT GAT GCC AAA TGC AAA AAG GAG CGG AAG AAG AAA
781 811
AAG AAA GTG ACC AAC ATA ATC TCA TTT GAT GAT GAG GAA GAT GAG CAG AAC TCT GGG GAC
841 871
GTG TTT AAA AAG ACA CCT GGG GCA GGG GAG AGC TCA GAG GAC AAC TCC GAC CGC TCC TCT
901 931
GTC AAT ATC ATG TCC GCC TTT GAA AGC CCC TTC GGG CCT AAC TCC AAT GGA AGT CAG AGC
961 991
AGC AAC TCA TGG AAA ATT GAT TCC CTG TCT TTG AAC GGG GAG TTT GGG TAC CAG AAG CTT
1021 1051
GAT GTG AAA AGC ATC GAT GAT GAA GAT GTG GAT GAA AAC GAA GAT GAC GTG TAT GGA AAC
1081 1111
TCA TCA GGA AGG AAG CAC AGG GGC CAC TCG GAG TCG CCC GAG AAG CCA CTG GAA GGG AAC
1141 1171
ACC TGC CTC TCC CAG ATG CAC AGC TGG GCT CCG CTG AAG GTG CTG CAC AAT GAC TCC GAC
1201 1231
ATC CTC TTC CCT GTC AGT GGC GTG GGC TCC TAC AGC CCA GCA GAT GCC CCC CTC GGA AGC
1261 1291
CTG GAG AAC GGG ACA GGA CCA GAG GAC CAC GTT CTC CCG GAT CCT GGA CTT CGG TAC AGT
1321 1351
GTG GAA GCC AGC TCT CCA GGC CAC GGA AGT CCT CTG AGC AGC CTG TTA CCT TCT GCC TCA
1381 1411
GTG CCA GAG TCC ATG ACA ATT AGT GAA CTG CGC CAG GCC ACT GTG GCC ATG ATG AAC AGG
1441 1471
AAG GAT GAG CTG GAG GAG GAG AAC AGA TCA CTG CGA AAC CTG CTC GAC GGT GAG ATG GAG
1501/501 1531
CAC TCA GCC GCG CTC CGG CAA GAG GTG GAC ACC TTG AAA AGG AAG GTG GCT GAA CAG GAG
1561 1591
GAG CGG CAG GGC ATG AAG GTC CAG GCG CTG GCC AGC TAT CTT TGC TAT TTT GTG AGG AGA
1621 1651
TTC TAA CCC CAC GTG AGA ACC ATG TGG TGG AGA AAT GGA GGG AGA GAG AAA TCC AAC AGT
1681 1711
TCC TGA TAG TCT CAT TTG AGC TCC TGG ATC CAG TCT TTC CTG AAG CTG TGT TTC CTC TGG
1741 1771
ACT TTT CAT GTA TGT GAG CCA ATA AAT TGC TTT CAT TCC TTG

Figure 3B: Nucleotide sequence of IREN

ATG AGC GGA TCA CAG AAC AAT GAC AAA AGA CAA TTT CTG CTG GAG CGA CTG CTG GAT GCA

61 91

GTG AAA CAG TGC CAG ATC CGC TTT GGA GGG AGA AAG GAG ATT GCC TCG GAT TCC GAC AGC

121 151

AGG GTC ACC TGT CTG TGT GCC CAG TTT GAA GCC GTC CTG CAG CAT GGC TTG AAG AGG AGT

181 211

CGA GGA TTG GCA CTC ACA GCG GCA GCG ATC AAG CAG GCA GCG GGC TTT GCC AGC AAA ACC

241 271

GAA ACA GAG CCC GTG TTC TGG TAC TAC GTG AAG GAG GTC CTC AAC AAG CAC GAG CTG CAG

301 331

CGC TTC TAC TCC CTG CGC CAC ATC GCC TCA GAC GTG GGC CGG GGT CGC GCC TGG CTG CGC

361 391

TGT GCC CTC AAC GAA CAC TCC CTG GAG CGC TAC CTG CAC ATG CTC CTG GCC GAC CGC TGC

421 451

AGG CTG AGC ACT TTT TAT GAA GAC TGG TCT TTT GTG ATG GAT GAA GAA AGG TCC AGT ATG

481 511

CTT CCT ACC ATG GCA GCA GGT CTG AAC TCC ATA CTC TTT GCG ATT AAC ATC GAC AAC AAG

541 571

GAT TTG AAC GGG CAG AGT AAG TTT GCT CCC ACC GTT TCA GAC CTC TTA AAG GAG TCA ACG

601 631

CAG AAC GTG ACC TCC TTG CTG AAG GAG TCC ACG CAA GGA GTG AGC AGC CTG TTC AGG GAG

661 691

ATC ACA GCC TCC TCT GCC GTC TCC ATC CTC ATC AAA CCT GAA CAG GAG ACC GAC CCC TTG

721 751

CCT GTC GTG TCC AGG AAT GTC AGT GCT GAT GCC AAA TGC AAA AAG GAG CGG AAG AAG AAA

781 811

AAG AAA GTG ACC AAC ATA ATC TCA TTT GAT GAT GAG GAA GAT GAG CAG AAC TCT GGG GAC

841 871

GTG TTT AAA AAG ACA CCT GGG GCA GGG GAG AGC TCA GAG GAC AAC TCC GAC CGC TCC TCT

901 931

GTC AAT ATC ATG TCC GCC TTT GAA AGC CCC TTC GGG CCT AAC TCC AAT GGA AGT CAG AGC

961 991

AGC AAC TCA TGG AAA ATT GAT TCC CTG TCT TTG AAC GGG GAG TTT GGG TAC CAG AAG CTT

1021 1051

GAT GTG AAA AGC ATC GAT GAT GAA GAT GTG GAT GAA AAC GAA GAT GAC GTG TAT GGA AAC

1081 1111

TCA TCA GGA AGG AAG CAC AGG GGC CAC TCG GAG TCG CCC GAG AAG CCA CTG GAA GGG AAC

1141 1171

ACC TGC CTC TCC CAG ATG CAC AGC TGG GCT CCG CTG AAG GTG CTG CAC AAT GAC TCC GAC

1201 1231

ATC CTC TTC CCT GTC AGT GGC GTG GGC TCC TAC AGC CCA GCA GAT GCC CCC CTC GGA AGC

1261 1291

CTG GAG AAC GGG ACA GGA CCA GAG GAC CAC GTT CTC CCG GAT CCT GGA CTT CGG TAC AGT

1321 1351

GTG GAA GCC AGC TCT CCA GGC CAC GGA AGT CCT CTG AGC AGC CTG TTA CCT TCT GCC TCA

1381 1411

GTG CCA GAG TCC ATG ACA ATT AGT GAA CTG CGC CAG GCC ACT GTG GCC ATG ATG AAC AGG

1441 1471

AAG GAT GAG CTG GAG GAG GAG AAC AGA TCA CTG CGA AAC CTG CTC GAC GGT GAG ATG GAG

1501 1531

CAC TCA GCC GCG CTC CGG CAA GAG GTG GAC ACC TTG AAA AGG AAG GTG GCT GAA CAG GAG

1561 1591

GAG CGG CAG GGC ATG AAG GTC CAG GCG CTG GCC AGA GAG AAC GAG GTG CTC AAA GTC CAA

1621 1651

CTG AAG AAA TAT GTA GGA GCT GTC CAG ATG CTG AAA AGA GAA GGT CAA ACA GCT GAA GTG

1681 1711

CCA AAT CTT TGG AGT GTT GAT GGA GAA GTT ACA GTA GCT GAA CAG AAG CCG GGA GAA ATT

1741 1771

GCT GAA GAA CTC GCA AGC TCC TAC GAA AGA AAG CTC ATC GAG GTG GCA GAG ATG CAT GGC

1801 1831

GAG CTG ATT GAG TTC AAC GAG CGC CTG CAC AGG GCC CTG GTA GCC AAG GAA GCC CTC GTG

Figure 4: Nucleotide sequence of IREN-10B splice isoform (cont. on next page)

1861 1891
TCC CAG ATG AGG CAG GAG CTC ATC GAT CTC CGG GGA CCG GTG CCT GGA GAT TTG AGT CAA
1921 1951
ACG TCC GAA GAC CAG AGT TTG TCG GAT TTT GAA ATA TCA AAC CGG GCG CTG ATC AAC GTC
1981 2011
TGG ATC CCC TCA GTG TTT CTC CGG GGC AAA GCA GCA AAT GCA TTC CAC GTG TAT CAG GTC
2041 2071
TAC ATC CGG ATA AAA GAC GAT GAA TGG AAT ATT TAT CGC CGG TAT ACA GAG TTC AGG AGT
2101 2131
TTG CAC CAC AAG TTA CAA AAC AAG TAC CCT CAA GTG AGG GCC TAC AAC TTC CCA CCC AAA
2161 2191
AAG GCC ATT GGA AAC AAG GAT GCC AAG TTT GTG GAG GAA CGG AGA AAG CAG CTC CAG AAT
2221 2251
TAC CTG CGC AGC GTC ATG AAC AAA GTC ATC CAG ATG GTC CCC GAG TTC GCT GCC AGC CCC
2281 2311
AAG AAG GAG ACC CTC ATC CAG CTG ATG CCC TTC TTC GTC GAC ATC ACC CCG CCC GGA GAG
2341 2371
CCT GTG AAC AGC CGG CCC AAA GCA GCT TCC CGC TTT CCC AAA CTG TCC CGG GGT CAG CCC
2401 2431
CGG GAG ACC CGC AAC GTG GAG CCC CAG AGC GGT GAC CTC TGA CCT CGA CAA AAC CGC AGC
2461 2491
CAC GGG CCC TGT GCG TGG CAC CAG CTG CGT CCA CCC CAG CCA CTG CCG CTG GCC CCT CAC
2521 2551
CTC AGC GTG ACA ACC ACG TCC CAC TGG TGA TCC TGA GAG CAC ACG ATT CCC AAC AGT TAC
2581 2611
ACA ACA CCC CGA TTA AAC TAA TCA GTC TTC GAG CCG CAT GAT ACC GTG ACC CGA GAG ACC
2641 2671
AAG GCA GCA CCT CGC TGG AGA GAC TGG GAC ACA CAG TCC TTC TGC TTC TGG GGT CTA CCC
2701 2731
TGG GCT GCA AGG GCT GTT CCT CCA CCT TCC TAT AGT TCA GGG CTG GCA GGA GGG TGG GCA
2761 2791
CCA GGT CAG GCT GGG TGC GCC ATG GTT GAG AGG CAA AGG TGA TCC CCT ATA TAG GAA GGT
2821 2851
TCA TGC AGA GCC AGC CTC TCC ACT CTT TCC CAT GTG GGG ACT AGA ATG ACT ATT AGC CTC
2881 2911
TTC CTT TGC TTT TTA AGG TTA TTA CCT GGC CTA ACC TAG GGA TGG CTG GCT GTG GGG GGG
2941 2971
GGG GGT GGG CAT GGT TCC TTT CAC TGC ATT TTC CAC CAA CAG TCA TTA GAC ACC TGG CAC
3001 3031
TGT CAC AGC TCA CTT TTC CAG AGG GAT ATT CCT GTG GCT TTG GCA AGG AGC CAT TAG TGA
3061 3091
TGT GCA ACT TGA GTT CAG AGA ACT TCC CCT ACC TCC CCC ATG GCT GGC TTC AGG AAG GAC
3121
CAG TGC CCT CCA TAG CCT G

Figure 4: Nucleotide sequence of IREN-10B splice isoform

ATG AGC GGA TCA CAG AAC AAT GAC AAA AGA CAA TTT CTG CTG GAG CGA CTG CTG GAT GCA
61 91
GTG AAA CAG TGC CAG ATC CGC TTT GGA GGG AGA AAG GAG ATT GCC TCG GAT TCC GAC AGC
121 151
AGG GTC ACC TGT CTG TGT GCC CAG TTT GAA GCC GTC CTG CAG CAT GGC TTG AAG AGG AGT
181 211
CGA GGA TTG GCA CTC ACA GCG GCA GCG ATC AAG CAG GCA GCG GGC TTT GCC AGC AAA ACC
241 271
GAA ACA GAG CCC GTG TTC TGG TAC TAC GTG AAG GAG GTC CTC AAC AAG CAC GAG CTG CAG
301 331
CGC TTC TAC TCC CTG CGC CAC ATC GCC TCA GAC GTG GGC CGG GGT CGC GCC TGG CTG CGC
361 391
TGT GCC CTC AAC GAA CAC TCC CTG GAG CGC TAC CTG CAC ATG CTC CTG GCC GAC CGC TGC
421 451
AGG CTG AGC ACT TTT TAT GAA GAC TGG TCT TTT GTG ATG GAT GAA GAA AGG TCC AGT ATG
481 511
CTT CCT ACC ATG GCA GCA GGT CTG AAC TCC ATA CTC TTT GCG ATT AAC ATC GAC AAC AAG
541 571
GAT TTG AAC GGG CAG AGT AAG TTT GCT CCC ACC GTT TCA GAC CTC TTA AAG GAG TCA ACG
601 631
CAG AAC GTG ACC TCC TTG CTG AAG GAG TCC ACG CAA GGA GTG AGC AGC CTG TTC AGG GAG
661 691
ATC ACA GCC TCC TCT GCC GTC TCC ATC CTC ATC AAA CCT GAA CAG GAG ACC GAC CCC TTG
721 751
CCT GTC GTG TCC AGG AAT GTC AGT GCT GAT GCC AAA TGC AAA AAG GAG CGG AAG AAG AAA
781 811
AAG AAA GTG ACC AAC ATA ATC TCA TTT GAT GAT GAG GAA GAT GAG CAG AAC TCT GGG GAC
841 871
GTG TTT AAA AAG ACA CCT GGG GCA GGG GAG AGC TCA GAG GAC AAC TCC GAC CGC TCC TCT
901 931
GTC AAT ATC ATG TCC GCC TTT GAA AGC CCC TTC GGG CCT AAC TCC AAT GGA AGT CAG AGC
961 991
AGC AAC TCA TGG AAA ATT GAT TCC CTG TCT TTG AAC GGG GAG TTT GGG TAC CAG AAG CTT
1021 1051
GAT GTG AAA AGC ATC GAT GAT GAA GAT GTG GAT GAA AAC GAA GAT GAC GTG TAT GGA AAC
1081 1111
TCA TCA GGA AGG AAG CAC AGG GGC CAC TCG GAG TCG CCC GAG AAG CCA CTG GAA GGG AAC
1141 1171
ACC TGC CTC TCC CAG ATG CAC AGC TGG GCT CCG CTG AAG GTG CTG CAC AAT GAC TCC GAC
1201 1231
ATC CTC TTC CCT GTC AGT GGC GTG GGC TCC TAC AGC CCA GCA GAT GCC CCC CTC GGA AGC
1261 1291
CTG GAG AAC GGG ACA GGA CCA GAG GAC CAC GTT CTC CCG GAT CCT GGA CTT CGG TAC AGT
1321 1351
GTG GAA GCC AGC TCT CCA GGC CAC GGA AGT CCT CTG AGC AGC CTG TTA CCT TCT GCC TCA
1381 1411
GTG CCA GAG TCC ATG ACA ATT AGT GAA CTG CGC CAG GCC ACT GTG GCC ATG ATG AAC AGG
1441 1471
AAG GAT GAG CTG GAG GAG GAG AAC AGA TCA CTG CGA AAC CTG CTC GAC GGT GAG ATG GAG
1501 1531
CAC TCA GCC GCG CTC CGG CAA GAG GTG GAC ACC TTG AAA AGG AAG GTG GCT GAA CAG GAG
1561 1591
GAG CGG CAG GGC ATG AAG GTC CAG GCG CTG GCC AGA GAG AAC GAG GTG CTC AAA GTC CAA

Figure 5: Nucleotide sequence of IREN-E splice isoform (cont. on next page)

1621 1651
CTG AAG AAA TAT GTA GGA GCT GTC CAG ATG CTG AAA AGA GAA GGT CAA ACA GCT GAA GTG
1681 1711
CCA AAT CTT TGG AGT GTT GAT GGA GAA GTT ACA GTA GCT GAA CAG AAG CCG GGA GAA ATT
1741 1771
GCT GAA GAA CTC GCA AGC TCC TAC GAA AGA AAG CTC ATC GAG GTG GCA GAG ATG CAT GGC
1801 1831
GAG CTG ATT GAG TTC AAC GAG CGC CTG CAC AGG GCC CTG GTA GCC AAG GAA GCC CTC GTG
1861 1891
TCC CAG ATG AGG CAG GAG CTC ATC GAT CTC CGG GGA CCG GTG CCT GGA GAT TTG AGT CAA
1921 1951
ACG TCC GAA GAC CAG AGT TTG TCG GAT TTT GAA ATA TCA AAC CGG GCG CTG ATC AAC GTC
1981 2011
TGG ATC CCC TCA GTG TTT CTC CGG GGC AAA GCA GCA AAT GCA TTC CAC GTG TAT CAG GTC
2041 2071
TAC ATC CGG ATA AAA GAC GAT GAA TGG AAT ATT TAT CGC CGG TAT ACA GAG TTC AGG AGT
2101 2131
TTG CAC CAC AAG TTA CAA AAC AAG TAC CCT CAA GTG AGG GCC TAC AAC TTC CCA CCC AAA
2161 2191
AAG GCC ATT GGA AAC AAG GAT GCC AAG TTT GTG GAG GAA CGG AGA AAG CAG CTC CAG AAT
2221 2251
TAC CTG CGC AGC GTC ATG AAC AAA GTC ATC CAG ATG GTC CCC GAG TTC GCT GCC AGC CCC
2281 2311
AAG AAG GAG ACC CTC ATC CAG CTG ATG CCC TTC TTC GTC GAC TGG ATC TCA CTT GTT TGG
2341 2371
AAA TGG CCG CGA TAG TTC ACG TGA GGA GTT CTC ATC CTC TTA GCG GCA TCC CCA TGG CCC
2401 2431
AGG GTG CAC GGG GGA ATT AGC CTC TCG CGG AGT CAT CAC GCA TCG ACT GAA TTC CCT GGT
2461 2491
GAA AAC TGA GTT AGC CAG TTG TTC CTA AGA TAC TCC TGA TGC TGA GAG TGT GAG CAG GAG
2521 2551
GCG CTG CCC CAT CCG CAA GTC AGT GTC CCC CAC CCC CTG CGG GGT CCA CAG CCC AGG CAT
2581 2611
CTC CGG TCC AGT GTT TCC CAA ACA TTC GCG TGC CGA ATT GTA AAA AGT GCA CGT TAA TGC
2641 2671
GAG CCT GTC GGT GTG ACA TGA ATC TCA GCC ATG CTG GTT GCC ATC AGT CAG CAC GGA GAG
2701 2731
AGA AAC CTT TTG TGC CTA ATT AGC ACG CAG AAC AGA ACA CAG GGT TCG ATT TAT GGA CTT
2761 2791
TTC AAA ACG AGA ATT TCA GTG GGA GAC TGT GGC AAA TGA CAC AGT GTT GAC ACT GGA ATT
2821 2851
TTG ACT ACA TGT TGG TCT AGA GCG GCC GCC ACC GCG GTG GAG CTC CAA TTC GT

Figure 5: Nucleotide sequence of IREN-E splice isoform

M V R R E R C R L D Q I P K V V S D S T I L V V K H E F
S K V G T F A L P L N T V K F N N V S C L E E P D S R *
G Q T L E Y L S T N V A V V K I S K G L F N A E E A Q
S C C A P S N T M G T S S T K M W S R S P G S S L A G
Q Q L L V L E F A Q S S R N T S K I K Q V T S M E L M
N I C T F R H Y A S L A N I P A I D H M S G P T E R K
N R A A W H S E G K L V V I G F D D R H G P G I E Q V
D F Q A Y I L D L F K S S S A E S E G S V E H S N E Q
K G F A Y A E W N A E I A F G S L D H W G D G E R V A
R G E I V S R S S P S L D D E P S V S A S H S L S D L
Q R A K K D Y F I T T I A D S F L D E P Y V P R L T A
F K V Q E V L V L V Q K K E S G N E S L S L L Q R L S
L E L A V G H M F S G P C E E P G N P K P P S A N K Y
L I Q A L R M D A D V E K D D N E E E V A D S T L R L
E A H G N G L E I L S Q K E N S F D K L D P L V L K C
R S G F K R L E N L S E E Q S N G D P H A G L A D V Y
L D L A H A A R I K L T R N D G Y V L N P L P M G A F
L S K S E W D S D E F D K S R S Q Y E D L R S M E E V
D D R K L L R S N S R P K G S Q K G G S G Y A N M Q R
A S S T Q R C M K T E L K D S S L N N D S S S R E E R

Figure 6: Deduced amino acid sequence of IREN

MVRRERCRLDQIPKVVSDSTILVVKHELPAESTWYLKYKPR
SKVGTFALPLNTVKFNNVSCLEEPDSRKNELQSIIHALKVE
GQTLEYLSTNVAVVKISKGLFNAEEAQKLETMEPRHIRENT
SCCAPSNTMGTSSTKMWSRSPGSSLAGYWLERDSIKGSTSR
QQLLVLEFAQSSRNTSKIKQVTSMELMVSAFQQVKLNVLRN
NICTFRHYASLANIPAIDHMSGPTERKGVSNESFDQKMIPV
NRAAWHSEGKLVVIGFDDRHGPGIEQVADSELLLDNDNQKE
DFQAYILDLFKSSSAESEGSVEHSNEQVGYRISREKAKLAP
KGFAYAEWNAEIAFGSLDHWGDGERVAQEELDDGWYKVMAQ
RGEIVSRSSPSLDDEPSVSASHSLSDLMVRHLFKNPFIPSS
QRAKKDYFITTIADSFLDEPYVPRLTALTKRREAIQVQFRG
FKVQEVLVLVQKKESGNESLSLLQRLRKVLAGIAYVEMFFD
LELAVGHMFSGPCEEPGNPKPPSANKERAILPSNRREVVPL
LIQALRMDADVEKDDNEEEVADSTLRNEEEVVNARARPDK*
EAHGNGLEILSQKENSFDKLDPLVLKEGQVAPRFYYREIL
RSGFKRLENLSEEQSNGDPHAGLADVVQKAKGAHTNKFTS
LDLAHAARIKLTRNDGYVLNPLPMGALTPEEDIVEFQAPR
LSKSEWDSDEFDKSRSQYEDLRSMEEKAGMALIYFPLAPG
DDRKLLRSNSRPKGSQKGGSGYANMQVEEHLSNQRPQSGQ
ASSTQRCMKTELKDSSLNNDSSSREEQVIGVQVVSKNPEP

Figure 7: Deduced amino acid sequence of IREN-10B splice isoform

MVRRERCRLDQIPKVVSDSTILVVKHELPAESTWYLKYKK
SKVGTFALPLNTVKFNNVSCLEEPDSRKNELQSIIHALKW
GQTLEYLSTNVAVVKISKGLFNAEEAQKLEIMEPRHIREP
SCCAPSNTMGTSSTKMWSRSPGSSLAGYWLERDSIKGSTR
QQLLVLEFAQSSRNTSKIKQVTSMELMVSAFQQVKLNVL*
NICTFRHYASLANIPAIDHMSGPTERKGVSNESFDQKMI
NRAAWHSEGKLVVIGFDDRHGPGIEQVADSELLDNDNQ
DFQAYILDLFKSSSAESEGSVEHSNEQVGYRISREKAKL
KGFAYAEWNAEIAFGSLDHWGDGERVAQEELDDGWYKVM
RGEIVSRSSPSLDDEPSVSASHSLSDLMVRHLFKNPFIP
QRAKKDYFITTIADSFLDEPYVPRLTALTKRREAIQVQF
FKVQEVLVLVQKKESGNESLSLLQRLRKVLAGIAYVEMF
LELAVGHMFSGPCEEPGNPKPPSANKERAILPSNRREVV
LIQALRMDADVEKDDNEEEVADSTLRNEEEVVNARARPD
EAHGNGLEILSQKENSFDKLDPLVLKEGQVAPRFYYREW
RSGFKRLENLSEEQSNGDPHAGLADVVQKAKGAHTNKFI
LDLAHAARIKLTRNDGYVLNPLPMGALTPEEDLVEFQAS
LSKSEWDSDEFDKSRSQYEDLRSMEEKAGMALIYFPLAL
DDRKLLRSNSRPKGSQKGGSGYANMQVEEHLSNQRPQSV
ASSTQRCMKTELKDSSLNNDSSREEQVIGVQVVSKNPW

Figure 8: Deduced amino acid sequence of IREN-E splice isoform

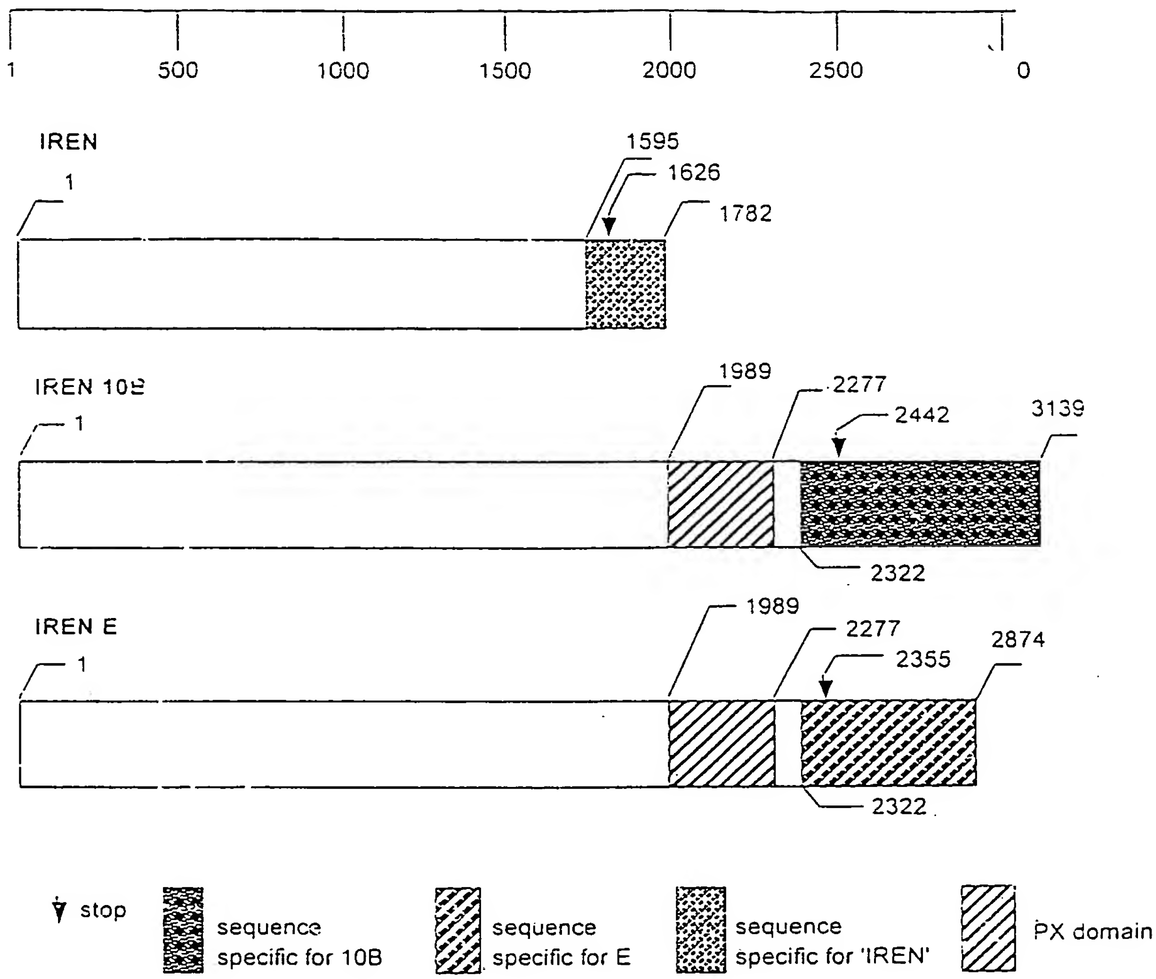
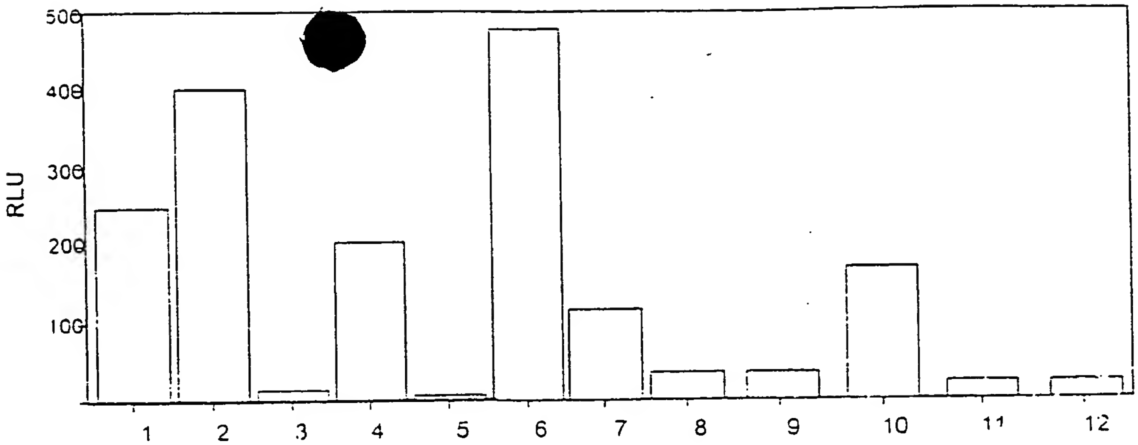
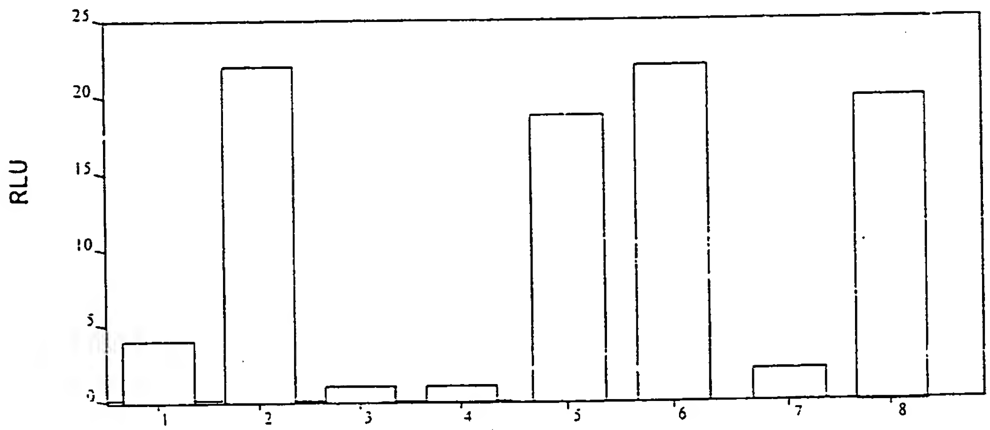

1
500
1000
1500
2000
2500
0
IREN
1
1595
1626
1782
IREN 10B
1
1989
2277
2442
3139
2322
IREN E
1
1989
2277
2355
2874
2322
stop
sequence specific for 10B
sequence specific for E
sequence specific for 'IREN'
PX domain

Figure 9



1 NIK (0.5 mcg)
2 NIK (0.5 mcg) + IREN
3 NIK + NEMO
4 NIK + NEMO + IREN
5 Control
6 NIK
7 NIK + IRENΔN
8NIK + NEMO1-309
9 NIK + NEMO + IRENΔN
10 p55
11 p55 + NIK mut
12 p55 + NIK mut + IREN



1 IKK-1
2 IKK-1 + IREN
3 IKK-1 + NEMO 1-309
4 IKK-1 + IREN + NEMO 1-309
5 IKK-1 + NEMO
6 IKK-1 + NEMO + IREN
7 IKK-1 + NIKmut
8 IKK-1 + NIKmut + IREN

Figure 10

Figure 11